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THE POTENCY AND DISTRIBUTION IN THE SERUM FRACTIONS OF ANTIVIRAL BODY OBTAINED BY IMMUNIZATION OF THE HORSE WITH VACCINIA VIRUS.

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THE decision to immunize a horse with vaccinia virus was taken with two main objects in view, firstly to secure an ample supply of antiviral serum for various studies, and secondly in order to have at hand, if necessary, a potent serum that might be employed, empirically at least, in cases of post-vaccinal encephalitis or virulent smallpox.

The present communication is concerned only with the distribution of antiviral body in the serum fractions (Section I), and with the results of various tests for estimating the potency of the serum in experimentally infected animals (Section II).

The available literature on the first of these questions will be referred to in the subsequent discussion. We may simply note here that our knowledge of this subject is incomplete, and that further information is desirable in the interest of improved concentration methods as applied to antiviral sera.

SECTION I.

Details of immunization of horse "Party."

Admitted to stables 10.v.29. Between 24.v.29 and 11.vii.29 received a course of immunization with formalized diphtheria toxin and a course of tetanus immunization consisting of 5 doses of formalized toxin. Throughout the subsequent treatment with vaccine lymph (calf-lymph kindly supplied by the late Dr. Blaxall, of the Government Lymph Establishment) the horse received routine doses of tetanus toxoid every two or three months.

Vaccination.—On 29.vii.29 a shaved area about 1 ft. square on the off-side of the neck was lightly scarified and a 1 in 10 dilution of glycerinated calf-lymph rubbed in thoroughly with a glass rod. Fifty hours after vaccination a considerable crop of papules appeared on the pink skin. On the fourth day some of the papules showed a brownish apex, indicating the drying up of vesicles. About 200 papules in all were counted, and there was evidence of considerable dermal infiltration.

Detachment of scabs was noted about the 18th day and was complete by the 28th day. After the 35th day the skin was apparently normal. Details of the subsequent treatment of the horse (hyperimmunization process), including dates and amounts of bleedings, will be found in annexed tables (Tables I and II). Subcutaneous and intravenous injections were followed by little disturbance of any kind and the temperature and pulse-rate were scarcely affected. On 7.vii.30, following an intramuscular dose of 50 c.c., an infected abscess resulted, which healed so rapidly after surgical treatment that it was considered safe to inoculate a dose of 100 c.c. into the muscle of the opposite side of the neck on 24.vii.30. Two days later the site of inoculation was swollen and œdematous, and the temperature rose to 104° F. Under an anæsthetic the swelling was opened, but after consciousness was regained it was found that the hindquarters were paralysed. The symptoms indicated some spinal injury, possibly from embolus in the lumbar region of cord, and the horse died on 27.vii.30.

TABLE I.—*Dates and Routes of Inoculation.*

Date.	Route.	Number of doses.	Total amount of lymph in c.c.
29.vii.29	Skin of neck	—	—
16.viii.29 to 14.x.29	Subcutaneous	8	10·5
21.x.29 to 12.xii.29	Intravenous	9	71
10.ii.30 to 24.ii.30	Ditto	5	31
10.iii.30 to 20.iii.30	,,	4	40
23.vi.30 to 7.vii.30	Intramuscular	4	130
Totals		30	282·5

TABLE II.—*Samplings and Bleedings during Hyperimmunization.*

Date.	Amount in litres.	Remarks.
29.vii.29	2	Control sample before vaccination.
16.viii.29	0·75	After skin vaccination.
21.x.29	4	After subcutaneous course.
16.xii.29 to 9.i.30	22	After first intravenous course (Batches 1 and 2, fractionated).
4.iii.30	2	After second intravenous course.
28.iii.30 to 4.iv.30	20	After third intravenous course (Batch 3, fractionated).
18.vii.30 to 17.vii.30	14	After final intramuscular course (Batch 4, fractionated).

Fractionation Methods Employed with Various Batches of Plasma.

Batch No. 1 (16.xii.29).—1000 c.c. plasma, 1160 c.c. distilled water, 30 gm. NaCl, 20 c.c. of 12 per cent. solution of $CaCl_2$ and 840 c.c. of a saturated ammonium sulphate solution (equivalent to 28 per cent. of saturation calculated on the total volume of fluid) were mixed. Euglobulin precipitate filtered off, pressed and dialysed. To 150 c.c. recovered from dialysing bags, 1 per cent. of NaCl was added. This preparation represented the *euglobulin fraction*, but as no steps were taken to remove associated pseudoglobulin from the euglobulin precipitate, it is certain that some pseudoglobulin was admixed with it.

To the filtrate from the first sulphate mixture enough saturated ammonium sulphate was added to bring the concentration of the salt to 46 per cent. saturation. Resulting pseudoglobulin precipitate was filtered off, pressed and dialysed. This solution represented the *pseudoglobulin fraction*, but contained residual euglobulin.

Batch No. 2 (6.i.30 and 9.i.30).—Method as for Batch No. 1, except that the dialysate (1500 c.c.) was divided into two portions. One litre was filtered after addition of 0·85 per cent. NaCl and 0·35 per cent. trikresol. The remaining 500 c.c. were diluted to 5000 c.c. with distilled water and the pH adjusted to 6·0 with acetic acid. After standing overnight the mixture was spun in a Sharples centrifuge. To the supernatant fluid ammonium sulphate was added to bring the concentration of the salt to 46 per cent. of saturation. Resulting precipitate filtered off, pressed and dialysed. Dialysate was spun in the Sharples centrifuge and 0·8 per cent. NaCl added

to the clarified solution. This reconcentrated or purified pseudoglobulin contained 15·6 per cent. protein.

Batch No. 3 (28.iii.30 to 4.iv.30).—Plasma from three bleedings was mixed and clotted by $CaCl_2$. Fibrin removed by filtration. Nine litres of serum were mixed with 4500 c.c. of distilled water and 5800 c.c. of saturated ammonium sulphate (equivalent to 30 per cent. saturation) and the resulting precipitate filtered off. The precipitate was mixed with 5000 c.c. of a solution of ammonium sulphate equivalent to 30 per cent. of saturation and was again filtered off, pressed and dialysed.

300 c.c. of euglobulin solution were thus obtained. This solution was diluted to 2000 c.c. with water, NaCl added to saturation and the mixture filtered to remove any residual pseudoglobulin. The precipitate was collected, pressed and dialysed. This preparation may be regarded as a *purified euglobulin solution.*

The sulphate filtrates collected during the process were mixed and ammonium sulphate added to 46 per cent. saturation. Resulting precipitate of pseudoglobulin was filtered off, pressed and dialysed. This preparation represents the first *pseudoglobulin fraction.* The filtrate from the last stage was saturated with ammonium sulphate and the resulting precipitate filtered, pressed and dialysed. This preparation represents the *albumin fraction.* 250 c.c. of the first pseudoglobulin fraction was diluted to 5000 c.c. with distilled water, the pH adjusted to 5·6 and the precipitate separated by filtration. An equal volume of saturated ammonium sulphate solution was added to the filtrate and the precipitate pressed and dialysed. This preparation represents the *purified pseudoglobulin,* and contained presumably a minimal amount of euglobulin. Gravimetric estimations of the protein in the four samples subsequently submitted to tests for potency were as follows: Purified euglobulin 8·8 per cent., first pseudoglobulin fraction 16·2 per cent., purified pseudoglobulin (reconcentrated) 14·6 per cent., albumin 8·5 per cent.

Batch 4 (15.vii.30 to 17.vii.30).—The plasma from this bleeding was dealt with in somewhat greater detail. Five different fractions were prepared, three containing euglobulin, one pseudoglobulin and one albumin.

Euglobulin A (dialysis).—300 c.c. of plasma dialysed 4 days against tap-water. The dialysate was made up to 600 c.c. with distilled water and pH adjusted to 4·5. Precipitate removed by Sharples, washed with small volume of distilled water and dissolved in 150 c.c. normal saline. Passed through Seitz. Protein percentage 0·85.

Euglobulin B (fraction insoluble in saturated sodium chloride).—300 c.c. plasma precipitated with an equal volume of saturated ammonium sulphate, sp. gr. 1·252. Precipitate shaken thoroughly and allowed to stand with 300 c.c. of saturated sodium chloride. The insoluble protein was filtered off, and after dialysis dissolved in 150 c.c. of normal saline. Passed through Seitz. Protein percentage 0·72.

Euglobulin C (34 per cent. saturation with ammonium sulphate).—300 c.c. plasma mixed with 155 c.c. saturated ammonium sulphate and after shaking allowed to stand for 2 hours. Filtered. Precipitate pressed and dialysed and finally dissolved in 150 c.c. saline. Passed through Seitz. Protein percentage 8·50.

Pseudoglobulin D (between 40 and 46 per cent. saturation with ammonium sulphate).—300 c.c. plasma mixed with 200 c.c. ammonium sulphate. Shaken and allowed to stand for 2 hours. Filtered. To filtrate 40 per cent. ammonium sulphate added to make 500 c.c. Ammonium sulphate further added to give 46 per cent. saturation. Precipitate filtered off, dialysed free from salt and dissolved in saline. Protein percentage 3·26.

Albumin E.—500 c.c. plasma + 500 c.c. water + 1170 c.c. saturated ammonium sulphate giving 54 per cent. Precipitate was filtered off and washed with 54 per cent. saturated ammonium sulphate to give a total volume of filtrate and washings of 2170 c.c. Increase to 60 per cent. gave so small a precipitate that the level was raised to 74 per cent. Precipitate dialysed and dissolved in saline. Protein percentage 4·2.

Distribution of the Total Protein in the Various Fractions.

As the fractions (*A–E*) of this batch were submitted to repeated potency tests (see later) it was necessary to secure further data which, in conjunction with the protein percentage of the suspension tested, would afford some quantitative expression for the distribution of the antibody in the various fractions. Dry weight estimations were accordingly made of the protein thrown out from the plasma of this batch by dialysis and by ammonium sulphate in ascending concentrations. The following data were thus obtained:

Total protein in 100 c.c. plasma, 7·9 gm.

Protein thrown out by dialysis (euglobulin), 7·6 per cent. of total.

,,	,,	30	per cent. of saturated ammonium sulphate,			17·5	per cent. of total.	
,,	,,	34	,,	,,	,,	29	,,	,,
,,	,,	40	,,	,,	,,	50·6	,,	,,
,,	,,	46	,,	,,	,,	60·7	,,	,,

From these data the following factors were ascertained and employed in subsequent quantitative potency estimations :

Euglobulin (A) in 100 c.c. plasma, 0·65 gm.

Pseudoglobulin (D) in 100 c.c. plasma, 4·2 gm.

Albumin (E) in 100 c.c. plasma, 3·05 gm.

" Euglobulin " C, precipitated by 34 per cent. of saturated ammonium sulphate (solution) containing a considerable admixture of pseudoglobulin, 2·3 gm.

Notes on methods of potency estimation.—Intradermal inoculation in shaved backs of rabbits ; dose injected in all cases 0·2 c.c. Glycerinated sheep-lymph used as test virus in various concentrations ; as a rule the dilution of virus decided upon was kept constant, and mixed in equal parts with the various serum dilutions to make a total volume of 0·2 c.c. The skin responses were measured daily with calipers and recorded as millimetres (diam.). When, as only rarely happened with this glycerinated virus, the response was not circular and circumscribed, the average of two diameters was taken. The test virus itself was frequently titrated on the same animal, so that in some experiments it was possible to estimate the potency of the serum or serum fraction in terms of the number of minimal infecting skin doses neutralized by 1 c.c. This method, however, as most workers have found (see especially Andrewes, 1929), fails to yield reliable absolute figures when the same materials are titrated on different animals. On the other hand, as the experiments detailed here show, comparative potency ratios of the same fractions proved remarkably constant when different rabbits and different test doses were used. To choose as basal figure the dilution of serum which effected complete neutralization would have given a quite inadequate idea of the strength of the serum or serum fraction. The choice of the serum dilution in which response to the test virus equalled that in the controls would have been more satisfactory had it not been for the fact that, as the dilution of a potent serum increased, the response of the test virus only very slowly increased and end-points were not always reached. It was decided, therefore, for comparative purposes to take cognizance of all the responses in a titration, and the method finally adopted in suitable cases was to subtract the sum of the cubes of the diameters of the lesions in any titration from the sum of the cubes of the diameters where only the virus was acting, the number of terms being the same in each. The difference so found was then expressed as a percentage, being in reality the percentage of the total possible volumetric response which was neutralized by the particular antiviral fluid in the undiluted state and in its ascending dilutions. The figure thus derived was divided by the known protein content in order to express the antibody-content per gramme of protein, and this latter figure was finally multiplied by the figure for the number of grammes of this fraction represented in the total protein (see above). The validity of this manipulation may, in certain particulars, be open to question. It was devised to meet the great difficulty of securing some quantitative expression for potency in comparative experiments. Experience with it suggests that for the future the potency of any anti-vaccinial serum may be best represented as a ratio in terms of a standard serum maintained in the dry state and titrated simultaneously on the same animal.

Abbreviations used in tables.—V., virus ; N.H.S., normal horse-serum (before immunization) ; N., *nil* or complete neutralization. The readings of diameters, at least during the critical days, are given in detail. In much published work on titrations of this kind precise protocols are frequently omitted and it is felt that the actual figures may be of interest to other workers in this field.

Potency of the Natural Serum of the Horse at Various Stages During Immunization.

Experiment 1.—Comparison of N.H.S. (29.vii.29), " A " (16.viii.29) and " B " (21.x.29). Date of test, 26.xi.29.

In this preliminary test the serum was kept constant while the virus varied. Calipers not used. Reading 4th day.

V (50) + N.H.S. ++++.	V (50) + A. +++.	V (50) + B. N.
V (500) + N.H.S. +++.	V (500) + A. +.	V (500) + B. N.
V (5000) + N.H.S. +.	V (5000) + A. N.	V (5000) + B. N.
V (50,000) + N.H.S. +.	V (50,000) + A. N.	V (50,000) + B. N.
V (500,000) + N.H.S. N.	V (50,0000) + A. N.	V (500,000) + B. N.

Remarks.—Definite appearance of antiviral body as the result of the primary cutaneous vaccination and very considerable increase following the first hyperimmunization course.

Experiment 2.—In view of suggestions which have been made that hyperimmunization with virus does not invariably result in an ever-increasing development of antibody, it was decided to test this point some time after the close of immunization, when samples from many bleedings would be available. All such samples of natural serum had been filtered and stored in the cold room. Samples tested (see Table II): "A" (N.H.S. 29.vii.29), "B" (16.viii.29), "C" (21.x.29), "D" (16.xii.29), "E" (6.i.30), "F" (4.iii.30), "G" (15.vii.30), and "H" (26.vii.30, day before death of horse). Date of test, 11.iii.31.

Experiment 2.

	Readings.			Readings.	
	5 days.	7 days.		5 days.	7 days.
V (100) + A	11	8	V (100) + F (1)	N	N
,, + A	13	8	,, + F (5)	1	2
,, + B (1)	4	5	,, + F (10)	4	4
,, + B (5)	8	5	,, + F (50)	12	9
,, + B (10)	8	6	,, + G (1)	N	N
,, + C (1)	N	N	,, + G (5)	N	N
,, + C (5)	N	N	,, + G (10)	N	N
,, + C (10)	1	N	,, + G (50)	1	N
,, + D (1)	N	N	,, + G (100)	5	6
,, + D (5)	Material lost.		,, + H (1)	N	N
,, + D (10)	6	6	,, + H (5)	N	N
,, + E (1)	1	1	,, + H (10)	N	N
,, + E (5)	10	4	,, + H (50)	5	N
,, + E (10)	9	8	,, + H (100)	6	7
,, + E (50)	5	4			

Remarks on Experiment 2.—The end-points were not reached in all cases, but the data show clearly that the very moderate rise in antibody following skin vaccination (B) is greatly enhanced as the result of the first hyperimmunization course by the subcutaneous route and the first intravenous course (C and D). The titrations of E and F following the second and third intravenous courses show a distinct falling off, possibly as the result of the large bleedings then taken. The final intramuscular course, however, again raises the antibody content to a high level.

By taking the cubes of the diameters (on the 5th day) and working out the neutralization percentage, the samples may be arranged in order of potency thus: C 99, G 98·5, H 96, B 79·7, F. 74, E 73·1. There is only a suggestion that the subcutaneous and intramuscular routes may be more effective in stimulating antibody formation than the intravenous. It seems highly

probable, as Laidlaw and Dunkin (1931) have observed in the hyperimmunization of dogs with distemper virus, that the early hyperimmunization after the initial stimulation from the primary virus infection may be sufficient to produce a maximal antibody yield.

Potency of Fractions from Various Bleedings.

Experiment 3: *Batch* 1.—(16.xii.29.) Comparison of natural serum "A" and concentrated serum (pseudoglobulin) "B." Protein percentage of latter, 14. Date of test, 16.i.30.

Experiment 3.

	Readings.				Readings.		
	2 days.	4 days.	8 days.		2 days.	4 days.	8 days.
V (25) + Sal. . .	17	21	20	V (25) + A (100) .	13	19	17
,, + Sal. . .	15	20	20	,, + B (1) . .	*22*	N	N
V (100) + Sal. . .	9	21	19	,, + B (10) . .	11	6	3
V (1000) + Sal. . .	5	11	14	,, + B (50) . .	7	11	6
V (10,000) + Sal. .	2	4	3	,, + B (100) .	10	14	11
V (100,000) + Sal. .	N	N	N	,, + B (500) .	10	16	14
V (25) + A (1) . .	14	6	N	,, + B (1000) .	12	19	18
,, + A (10) . .	10	15	14	,, + N.H.S. (1) .	20	25	23
,, + A (50) . .	10	17	15	,, + N.H.S. (10) .	13	22	19

Remarks on Experiment 3.—Test dose of virus has been fixed somewhat high. Attention is drawn to the high reading on second day with undiluted B, due simply to the high content of horse protein in the sample. It has disappeared by the fourth day. Normal horse-serum has no neutralizing value.

Analysis (by cubes) of 4th day readings. N.P.= Percentage neutralized.

	Total response.	N.P.	Antibody per gm.	Antibody in 100 c.c.
A. .	15363 .	63·9 .	7·9 (= 63·9/8) .	63·9
B. .	4291 .	89·9 .	6·4 (= 89·9/14) .	26·8 (= 6·4 × 4·2

Possible response $4 \times 22^3 = 42592$.

Experiment 4: *Batch* 3.—Comparison of two samples of pseudoglobulin. "A" a less refined sample of protein content 16·2 and "B" a more refined sample of protein content 14·6 (freed from euglobulin). This experiment is entered for two reasons—firstly to show the superiority of the more refined product, and secondly to demonstrate that the influence of high protein content

Experiment 4.

	Readings.				Readings.			
	2 days.	5 days.	6 days.	7 days.	2 days.	5 days.	6 days.	7 days.
V (80) + Sal. .	30	30	30	24	V (80)+ A (500) .	20	20	20	20
V (800) + Sal. .	15	16	12	13	,, + B (1) .	3	*26*	N	N
V (8000) + Sal. .	5	5	3	N	,, + B (10) .	5	*22*	N	N
V (80 + A (1) .	2	*35*	N	N	,, + B (50) .	10	*29*	10	4
,, + A (10) .	12	*27*	N	N	,, + B (100) .	17	23	15	8
,, + A (50) .	17	*24*	14	6	,, + B (500) .	14	20	16	15
,, + A (100) .	20	22	18	17					

Analysis by cubes (7th day reading):

	Total response.	N.P.	Antibody per gm.
A.	13129	81·0	5·0
B.	3951	94·2	6·4

Possible response 69120 $= 5 \times 24^3$.

may not manifest itself locally till after the third or fourth day, when usually the crucial readings are taken. These acute œdematous reactions may disappear entirely in 24 hours, leaving only the virus effect. Date of test, 26.iii.30.

Experiment 5: *Batch* 3.—Comparison of natural serum ("A"), pseudoglobulin ("B"), euglobulin ("C"), and albumin ("D"). Date of test, 6.vi.30.

Experiment 5.

	Readings.			Readings.	
	4 days.	6 days.		4 days.	6 days.
V (80) + N.H.S.	23	22	V (80) + B (1)	N	N
V (800) + Sal.	14	17	,, + B (10)	N	N
V (8000) + Sal.	5	3	,, + C (1)	N	N
V (80,000) + Sal.	N	N	,, + C (10)	6	8
V (80) + A (1)	N	N	,, + D (1)	22	20
,, + A (10)	1	N	,, + D (10)	26	26

Remarks.—End-points not reached, except in the case of the albumin, which exhibits no neutralizing value. It is clear that the euglobulin sample has considerable neutralizing power, and it was decided to titrate both it and the pseudoglobulin down to 1 in 500 (see Experiment 6).

Experiment 6: *Batch* 3.—Further titration of pseudoglobulin and euglobulin (here denoted "A" and "B") tested in Experiment 5.

"A" protein content, 15·6, "B" 8·8. Date of test, 12.vi.30.

Experiment 6.

	Readings.				Readings.		
	2 days.	4 days.	6 days.		2 days.	4 days.	6 days.
V (80) + N.H.S.	10	27	30	V (80) + A (100)	3	15	17
,, + N.H.S.	11	30	28	,, + A (500)	6	25	20
V (800) + Sal.	1	11	17	,, + B (1)	3	N	N
V (8000) + Sal.	5	17	17	,, + B (10)	8	5	7
V (80) + A (1)	2	N	N	,, + B (50)	9	12	8
,, + A (10)	2	N	N	,, + B (100)	6	16	11
,, + A (50)	3	2	N	,, + B (500)	11	23	17

Remarks.—On this occasion the high protein content of the pseudo-globulin sample did not complicate the virus response. Though the two fluids tested do not appear to differ greatly in potency, analysis reveals a considerably higher content of antibody per gramme of protein in the euglobulin sample.

Analysis (4th day reading):

	Total response.	N.P.	Antibody per gm.	Antibody in 100 c.c.
A.	19008	80·6	5·1	21·4
B.	18116	81·5	9·2	5·98

Possible response $98415 = 5 \times 27^3$.

Concentration of antibody in "A" : concentration in "B" = 1 : 1·8.

Total antibody in "A" : total in "B" = 3·5 : 1.

Experiments 7–11 : *Batch* 4.—The serum fractions A–E prepared from this final bleeding have been submitted to repeated comparative potency tests, and the readings of five of these tests in which the virus used varied in concentration from 1 in 60 to 1 in 150 are appended. In two of the tests the natural serum (F) is included.

Dates of the five tests.—5.xii.30, 13.xii.30, 10.iv.31, 17.iv.31, 28.iv.31.

Simple inspection of the readings in the various tests reveals the superiority of the "euglobulin" sample "C" which was obtained by 34 per cent. saturation with ammon. sulph. Next in order of potency came usually the pseudoglobulin, followed by the classical euglobulins. Here again the albumin sample ("E") is clearly devoid of neutralizing power, and appears indeed to enhance the severity of the virus response.

Experiment 7.

	Readings.				Readings.		
	3 days.	5 days.	8 days.		3 days.	5 days.	8 days.
V (60) + N.H.S. (1) .	19	21	19	V (60) + B (10) . .	15	16	11
V (600) + N.H.S. (1) .	11	12	9	,, + B (50) . .	17	17	15
V (6000) + N.H.S. (1) .	3	6	4	,, + C (1) . .	N	2	N
V (60,000) + N.H.S (1)	1	2	2	,, + C (10) . .	2	3	4
V (600,000) + N.H.S (1)	N	N	N	,, + C (50) . .	5	5	4
V (60) + A (1) . .	9	9	6	,, + D (1) . .	3	3	1
,, + A (10) . .	16	18	12	,, + D (10) . .	8	11	6
,, + A (50) . .	17	20	15	,, + D (50) . .	15	16	11
,, + B (1) . .	6	8	6				

Experiment 8.

	Readings.				Readings.		
	3 days.	4 days.	7 days.		3 days.	4 days.	7 days.
V (80) + N.H.S. (1) .	14	18	8	V (80) + D (1) . .	5	4	2
V (800) + N.H.S. (1) .	8	15	15	,, + D (10) . .	8	12	11
V (8000) + N.H.S. (1) .	7	9	16	,, + D (50)' . .	11	10	6
V (80) + C (1) . .	N	N	N	,, + E (1) . .	17	20	19
,, + C (10) . .	N	N	N	,, + E (10) . .	18	20	18
,, + C (50) . .	7	9	4	,, + E (50) . .	20	21	21
,, + C (100) .	11	14	5				

Experiment 9.

	Readings.				Readings.		
	3 days.	4 days.	6 days.		3 days.	4 days.	6 days.
V (100) + Sal. . .	21	21	16	V (100) + C (1) . .	2	4	4
V (1000) + Sal. . .	8	8	6	,, + C (5) . .	4	3	5
V (10,000) + Sal. .	3	1	N	,, + C (10) .	7	7	6
V (100,000) + Sal. .	N	N	N	,, + C (50) .	5	5	4
V (100) + A (1) . .	6	7	6	,, + C (100) .	6	7	4
,, + A (5) . .	11	7	6	,, + D (1) . .	3	6	4
,, + A (10) .	8	9	8	,, + D (5) . .	5	6	4
,, + B (1) . .	6	6	5	,, + D (10) .	11	11	11
,, + B (5) . .	12	13	9	,, + D (50) .	10	11	10
,, + B (10) .	13	16	12				

Experiment 10.

	Readings. 3 days.	4 days.		Readings. 3 days.	4 days.
V (150) + Sal.	11	10	V (150) + C (500)	5	4
,, + Sal.	11	10	,, + D (1)	1	N
,, + Sal.	11	10	,, + D (10)	2	1
,, + A (1)	5	5	,, + D (50)	4	3
,, + A (10)	7	7	,, + D (100)	7	5
,, + A (50)	8·5	8·5	,, + D (500)	9	10
,, + A (100)	10	10	,, + F (1)	1	N
,, + A (500)	9	8·5	,, + F (10)	4	1
,, + C (1)	1	N	,, + F (50)	6	6
,, + C (10)	1	1	,, + F (100)	6	6
,, + C (50)	1	3	,, + F (500)	10	9
,, + C (100)	4	4			

Experiment 11.

	Readings. 3 days.	4 days.		Readings. 3 days.	4 days.
V (100) + Sal.	9·5	10	V (100) + C (1000)	7·5	3
,, + Sal.	9·5	8·5	,, + D (1)	N	N
,, + A (1)	6	5·5	,, + D (10)	1	N
,, + A (10)	7	5	,, + D (50)	7	6
,, + A (50)	9	7	,, + D (100)	9·5	8
,, + A (100)	9·5	8·5	,, + D (500)	11	10·5
,, + A (500)	11	10·5	,, + D (1000)	11	9
,, + A (1000)	11	11	,, + F (1)	1	1
,, + C (1)	1	N	,, + F (10)	5	5
,, + C (10)	4	4	,, + F (50)	6	7
,, + C (50)	4	2	,, + F (100)	6·5	7
,, + C (100)	5·5	4	,, + F (500)	9	10
,, + C (500)	4·5	1	,, + F (1000)	9	7·5

Analysis by cubes of data in Experiments 7–11 (figures for total responses omitted):

		N.P.	Antibody per gm.	Antibody in 100 c.c.
Experiment 7 (5th day reading)	A.	47·6	56	36·4
	B.	65·7	91	..
	C.	99·4	11·7	26·9
	D.	80·3	24·6	103·3
Experiment 8 (4th day reading)	C.	90·6	10·6	24·3
	D.	89·9	27·5	115
Experiment 9 (4th day reading)	A.	94·9	111	72·1
	B.	76·5	106	..
	C.	98	11·5	26·4
	D.	91·6	28	117·6
Experiment 10 (3rd day reading)	A.	57·7	67·8	44·0
	C.	97·1	11·4	26·2
	D.	82·7	25·3	106·2
Experiment 11 (3rd day reading)	A.	42·7	50·2	32·6
	C.	97·2	11·4	26·22
	D.	59	18	75·6

If, as in Experiment 6, we compare the antibody contents per grm. of protein of the euglobulin and pseudoglobulin fractions (A and D), we find they stand to each other in the ratios 2·2 : 1, 3·9 : 1, 2·6 : 1 and 2·7 : 1, while the figures for the absolute amounts per 100 c.c. of serum are in the ratio 1 : 2·8, 1 : 1·6, 1 : 2·4 and 1 : 2·3. The agreement between the ratios yielded by the different experiments is good, and it is clear that while the concentration of antibody per gm. of protein in the euglobulin (dialysis) fraction is 2–3 times greater than that in the pseudoglobulin, this advantage is more than balanced by the much higher absolute amount of protein represented by pseudoglobulin in the unit volume of serum.

Brief Discussion of Data in Section I.

The antiviral body present in antivaccinial serum was first detected by Sternberg in 1892, and so long ago as 1899 Béclère, Chambon and Menard attempted to locate the position of this body among the serum proteins. They concluded that the antibodies were associated with the globulins only, and not at all with the albumin—a result which Findlay (1931) has recently confirmed. The data presented in this paper also support this conclusion. On the other hand, Henseval (1919) found that all the serum fractions, including the albumin, possessed some degree of antiviral activity. Large scale methods of concentrating sera, particularly antitoxic sera, have during the past fifteen years increased greatly in precision, so that quantitative comparisons between the older and the more modern findings are rendered of somewhat doubtful value. In antitoxic sera practically all the antitoxin is believed to be associated with the pseudoglobulin fraction, and concentration methods are directed towards the purification and concentration of this fraction. The study of certain antiviral sera from this point of view has revealed a different state of affairs. Hartley (1914) found that in anti-rinderpest serum the euglobulin fraction of the bovine serum contained all the antiviral body. In antipoliomyelitis serum prepared in the horse Morgan and Fairbrother (1930) find the antibody located, in highest concentration at least, in the euglobulin fraction, but its presence in the pseudoglobulin was also apparent. On the other hand, Weyer, Park and Banzhaf (1929), applying the ordinary concentration methods used for antitoxic sera to antipoliomyelitis serum, locate the antibody in what would appear to be, from its method of preparation, the pseudoglobulin fraction. In distemper antiserum prepared in the dog Laidlaw and Dunkin (1931) are satisfied that the major part of the antiviral content is associated with that small fraction of globulin known as the Felton fraction near the border-line of the salt precipitation limits of euglobulin and pseudoglobulin. With this fraction the effective antibody in antipneumococcus serum is, according to Felton, mainly associated. In the present study the chemical isolation of this fraction has not been attempted, because the potency tests have shown clearly that any method of concentration which excluded the pseudoglobulin would involve a serious loss of antibody. The classical euglobulin fraction does indeed contain per gramme of protein a considerably higher content of antibody than an equivalent amount of pseudoglobulin, but this higher concentration is more than compensated by the higher absolute amount of pseudoglobulin in the natural serum.

SECTION II.

Protective and Therapeutic Experiments with Antivaccinial Serum from the Horse.

Methods of standardizing antivaccinial serum which are based on direct neutralization tests in the rabbit dermis are undoubtedly the most readily applicable when quantitative comparative data are required. They tell us little, however, with regard to the possible value of the antiviral serum as a therapeutic agent. We record, therefore, a brief series of experiments in which attempt was made to ascertain how far such serum, when given intravenously, was capable (1) of preventing the growth of vaccinia virus directly implanted on skin surface and in dermis, and (2) of influencing the temperature reaction and the development of specific organic lesions.

Experiment 12.—Effect of antivaccinial serum intravenously administered on cutaneous and dermal responses in rabbits with calf lymph.

The scheme of the experiment was as follows:

27.i.30: Two rabbits ("A," "B") received 5 c.c. and 10 c.c. respectively of concentrated antivaccinial serum intravenously.

28.i.30: "A" and "B" received 0·2 c.c. of 1 in 5 calf lymph rubbed into skin of half-back immediately after shaving and also three dermal inoculations into skin of half-back shaved the previous day. The doses were 0·2 c.c. of dilutions 1 in 25, 1 in 250 and 1 in 2500 of centrifuged calf-lymph.

28.i.30: "C" and "D" received 5 c.c. and 10 c.c. respectively of serum intravenously and were simultaneously vaccinated as above.

28.i.30: "E," "F," "G," "H," "I," "J," "K" and "L" were vaccinated as above.

29.i.30: "E" and "F" received 5 c.c. and 10 c.c. of serum intravenously.

30.i.30: "G" and "H" received 5 c.c. and 10 c.c. of serum intravenously.

Thus two animals received serum on the day before vaccination, two simultaneously with vaccination, two on day after vaccination, two 48 hours after vaccination, while three ("I," "J," "K") received no serum. The readings at 24 hours, 3 days, 6 days and 10 days are appended.

Abbreviations.—I.D., intradermal; C., cutaneous reaction; N., no reaction. Figures under I.D. represent diameters of lesion in millimetres.

Experiment 12.

Rabbit.	Readings. 24 hours. I.D.	C.	3 days. I.D.	C.	6 days. I.D.	C.	10 days. I.D.	C.
A	1, 2, 5	N	N, 4, 6	N	N, 4, 5	N	N, 2, 3	N
B	N, 2, 4	N	N, 3, 5	N	N, 2, 4	N	N. 2, 3	N
C	N, N, 6	N	N, 10 10	N	N, 6, 9	N	N, 5, 7	N
D	N, N, 4	N	N, 6, 18,	N	N, 11, 15	N	N, 8, 11	N
E	5, 5, 8	N	1, 6, 15	+++	N, 2, 7	+++	N, N, 6	++
F	N, N, 5	N	N, N, 3	+	N, N, N	+++	N, N, N	+
G	N, 6, 7	N	2, 7, 10	++	N, 3, 6	+++	N, 2, 2	++
H	N, 3, 8	N	7, 11 18	+	5, 11, 13	++	N, 10, 12	++
I	N, 6, 7	N	9, 15, 17	++	9, 13, 14	+++	5, 11, 11	++
J	1, 4, 8	N	5, 7, 9	++	1, 4, 6	++	N, N, 2	++
K	N, 3, 5	N	4, 5, 8	++	4, 6, 9	++	1, 1, 4	+

Remarks on Experiment 12.—Complete abeyance of cutaneous response occurs only when the intravenous dose of antiviral serum is given 24 hours before or simultaneously with vaccination. When given 24 hours' start or longer, skin vaccination runs a fairly normal course. Complete abeyance of

the dermal responses is in no case obtained, but there is a very striking difference in the intensity of the responses to ascending doses of virus according as serum is given or not. It will be noted, for example, that in "A," "B," "C," "D," "E" and "F" there is no response at any time to the 1 in 2500 dose. If we take the 6th day reading of the dermal responses and cube the diameters, we get the following data :

Average response to 1 in 2500 in 8 receiving serum, 0·21.
" " in 3 controls 97·3.
Average response to 1 in 250 in 8 receiving serum, 110·5.
" " in controls 438·9.
Average response to 1 in 25 in 8 receiving serum, 389.
" " in controls 884.

One animal, F, which received serum 24 hours after vaccination, exhibited practically no dermal response.

The Influence of Antivaccinial Serum Intravenously Introduced on the Course of the Disease Produced in Rabbits by Vaccinia of Neurotesticular Origin given Intravenously.

The production of generalized lesions in the organs of rabbits after administration of potent strains of virus (especially neurotesticular virus) is now well recognized since the work of Douglas, Smith and Price (1929). Employing a strain of neurovaccine Andrewes (1929) showed that the occurrence of generalized lesions on skin following intradermal inoculation of the virus could be prevented by small doses of antiviral serum given intravenously. When the virus was introduced intravenously larger doses of serum were necessary to prevent generalized skin-lesions. Experiment 13 and the two following were designed, not so much with the object of titrating an antiviral serum by its effect on skin-lesions, but rather with a view to obtaining some measure of its influence on the general symptom-complex and pathology of the disease induced by the intravenous injection of neurotesticular vaccine. Before commencing these experiments it was anticipated that a death criterion might be possible, as in a previous series of animals inoculated intravenously with this type of virus (Ledingham and Barratt, 1929) 8 out of 12 animals similarly and simultaneously inoculated died in the first ten days. These deaths, particularly the earlier ones, were due to intensive hæmorrhagic lesions in the lungs and elsewhere, and were generally ushered in by a sudden fall of temperature to subnormal levels. It proved, however, that with the particular virus used in these experiments, though of very high potency by dermal titration, the capacity to produce hæmorrhagic lesions of such a character as to lead to a fatal issue was not marked. Resort therefore had to be made to recording temperature and weight during the acute phase, and to analyse the specific pathological findings in the various tissues after sacrificing the animals when normal temperature had been established.

Experiment 13.—Six rabbits received each 2·5 c.c. intravenously of a 1 in 12 dilution of testicular vaccine whose *M.I.D.* by dermal inoculation was 0·2 c.c. of a 1 in 100 million dilution. Three of them (A, B, C) received simultaneously in opposite ear 5 c.c. of antivaccinial serum, while the other three (D, E, F) received a similar quantity of normal horse-serum. The initial weights of the animals ranged from 50 to 60 oz.

Annexed are the temperature records, pyrexial phases being italicized :

Experiment 13.—*Temperature Readings.* (*Date of Injection of Virus and Serum, October 24th.*)

Rabbit.	October.								November.				
	24th.	25th.	26th.	27th.	28th.	29th.	30th	31st.	1st.	2nd.	3rd.	4th.	5th.
A	102·8	103	103	103	102·8	103	102·8	103	103	103	103·2	..	..
B	102·6	103	103·2	103·4	103·6	*104*	*104*	*105*	*104·4*	103·6	103	103·2	..
C	102·6	102·4	102·4	102·8	103	102·6	103	103·6	103·4	103	102·4	102·6	102·8
D	102·2	102·6	102·6	*106·4*	*105·8*	*106·2*	*105·6*	*104·4*	*104·4*	*103·8*	103	..	
E	102·4	102·4	103	*105·6*	*106*	*106*	*105·8*	*105·4*	*105·2*	*104*	*104·6*	103	..
F	102·8	103·2	103·2	*106*	*106·4*	*103·6*	*105·6*	*104·4*	*104*	*104*	*104·2*	103·4	103

The weight variations are omitted. On the 10th, 11th and 12th days the animals were sacrificed in pairs and the lesions recorded. They are summarized below :

Rabbit.	Eyes.	Lips.	Tongue.	Ears.	Skin.	Fascia.	Lungs.	Liver.	Adrenal.	Spleen.	Testes.	Ovaries and uterus.
A	N	N	N	N	N	N	+	N	N	N	–	N
B	N	N	N	N	N	N	+	N	N	N	N	–
C	N	N	N	N	N	N	+	?	?	N	+	+
D	++	++	+	++	+	+	+++	N	+	N	–	–
E	N	+	N	N	++	++	++++	N	++	++	–	N
F	N	+	N	+++	++	++	+++	?+	?++	N	–	++

Remarks.—Only in those animals which received the normal horse-serum is a pyrexial phase observed, commencing sharply on the 3rd day and lasting 7–8 days. One of the animals which received antiviral serum did indeed exhibit a brief pyrexial phase commencing on the 5th day and lasting 3–4 days. This late pyrexia is illustrated further in the next experiment.

With regard to the findings at autopsy the tables are self-explanatory. The animals which received specific serum showed practically no specific lesions except in the lungs, which on careful examination revealed a few characteristic pocks on the pleural surface. In the belief that the administration of antiviral serum a few hours before the dose of vaccinia might even keep lung lesions in check, the next experiment was performed.

Experiment 14.—Six rabbits (weights ranging from 43–47 oz.) received on 21.xi.30 each 2 c.c. of same testicular virus. A, B and C received 2 hours before injection of virus 5 c.c. each of antivaccinial serum intravenously ; D, E and F received no serum. Temperatures are recorded below :

Experiment 14.—*Temperature Readings.* (*Virus Inoculated November 21st.*)

Rabbit.	November.										December.			
	20th.	21st.	22nd.	23rd.	24th.	25th.	26th.	27th.	28th.	29th.	30th.	1st.	2nd.	3rd.	4th.
A	103	103	103	103·2	103	102·4	103·6	103·2	*106*	*105·6*	*105·4*	103	102·8	103	103
B	103	103·2	103	103·6	103·4	102·8	103·4	103·4	*105·4*	*104·2*	*104·8*	103·2	103·2	102·8	103
C	102·8	103	103	103·6	103·2	103·2	103	103·4	*105·4*	*103·6*	*104*	103·4	103	103	103
D	103·2	103·2	103·2	103	*106*	*105·2*	*105·6*	*105·6*	*105*	*104·4*	*103·8*	*103·6*	103·2	103	103
E	102·6	103	102·8	103·6	*107·2*	*105·6*	*105·6*	*105·6*	*105·4*	*104·2*	*104·4*	103	103·6	103·4	103·2
F	103·2	103	102·8	103·8	*105·8*	*105·4*	*105·4*	*105·6*	*105·6*	*104*	*103·6*	103	102·8	102·8	102·8

All six animals were killed by injection of air on the 13th day. The pathological findings are summarized below :

Rabbit.	Eyes.	Lips.	Tongue.	Ears.	Skin.	Fascia.	Lungs.	Liver.	Adrenal.	Spleen.	Testes.	Ovaries and uterus.
A	N	N	N	N	+	N	N	N	N	N	N	–
B	N	N	N	N	+	N	N	N	N	N	–	N
C	N	N	N	+	?1	N	+	N	N	N	–	N
D	N	N	N	++	++	+	++++	?+	+	N	-	+
E	N	+	N	++	++++	++	++++	N	++	N	–	N
F	N	N	N	++	+	+	+++	N	?N	N	++	–

Remarks.—The effect of administering the antiviral serum 2 hours before injection of virus has been to reduce lung lesions almost to zero. It will be noted, however, that in the skin of the back two of the animals receiving serum presented each a single large pock. It is possible that the predilection of pocks for the skin and fascia of the back may be due to some extent to handling. The lung lesions in the control animals were extremely severe, there being numerous pocks both on the pleural surface and in the lung substance. With regard to temperature, the controls showed a pyrexial phase of 7–8 days' duration, starting on the 3rd day, while the animals receiving serum passed through a lower-grade pyrexia of only 3 days' duration, starting on the 7th day.

The mechanism underlying this late brief pyrexial phase in animals receiving serum is not clear.

Experiment 15.—In the final experiment of this type an attempt was made to ascertain what effect on temperature and course might be expected to follow the intravenous administration of serum immediately after the temperature was found to be raised. A larger dose (3 c.c.) of virus was given on this occasion in the hope that a death criterion of potency might be possible. Nine rabbits received this dose intravenously on 19.i.31. As it happened, the abrupt rise of temperature took place in seven of the animals on the second day. On that day (21.i.31) 3 of these received each 7 c.c. of antiviral serum intravenously, while 3 received the same quantity of normal horse-serum. The remaining 3 animals received no serum.

The initial weights of the animals ranged from 40–50 oz.

The temperature readings are recorded below.

	January.													
Rabbit.	18th.	19th.*	20th.	21st. M.	21st. E.	22nd. M.	22nd. E.	23rd. M.	23rd. E.	24th.	25th.	26th.	27th.	28th.	29th.
A	103	102·8	103	*106*	*106·6*	*104·4*	*104·4*	*106·4*	*106·8*	*104·6*	*105·4*	*104·6*	*104*	*104·2*	*104·4*
B	102·8	103	103	*105·8* A.S.	*106*	*104·4*	*105*	*104·6*	*104·2*	*105·4*	*105*	*105*	103·4	103	103·4
C	103	102·8	103·4	*105·6* A.S.	*106·4*	*104*	*105*	*106·2*	*106·2*	101·6	97·6	+	..	..	..
D	103	103	103·8	*105·8* N.H.S.	*106·4*	*105·2*	*105·8*	*105*	*105·2*	101	+	..	..	..	..
E	102·8	102·8	103·2	*106·2* N.H.S.	*107·4*	*104·6*	*107·2*	*105·6*	*106*	*105*	*105·8*	*105*	*104·2*	*105*	*104*
F	103	103	103·4	*105·2* N.H.S.	*106·6*	103·2	103·8	*106·4*	*105·8*	*105·2*	103·2	103	103	103·4	102·6
G	102·4	102·6	103	103	103·4	*105*	*105·4*	*105*	*105·8*	*105·2*	*106*	*105·2*	*104·6*	*104·2*	103·2
H	103·2	103·4	103·2	103	*104·6*	*104*	*104·4*	*105·2*	*106*	*105*	*105·2*	*105·6*	*104·8*	*104*	103
I	103	102·8	103·2	*105·4*	*106*	*105·2*	*105·2*	*105·4*	*105*	101·4	98	+	..	..	..

* Inoculation of virus.

It will be noted that a marked fall of temperature occurred on the day following the injection of antiviral serum (A.S.) in rabbits A, B and C, but it was only temporary. In rabbit C the temperature rose, only to fall suddenly to subnormal level with fatal issue.

In rabbit D, which received normal horse-serum, the temperature fell only slightly, and two days later a fall to subnormal level occurred with fatal issue. In E and F the injection of normal horse-serum also produced a fall of temperature, but only temporarily. Of the controls which received no serum one (I) succumbed early with sudden fall to subnormal temperature, while the other two (G and H) ran the usual pyrexial course, lasting 7 days.

Post-mortem examination of the three animals which succumbed on the 6th and 7th days revealed the cause of death in all three to be hæmorrhagic œdema of the lungs. Confluent vaccinial lesions were present with profound œdema, and large portions of lung were black owing to hæmorrhage. Hæmorrhagic lesions were present elsewhere, particularly in the fascia over the muscles of the back. Apart from the immediate effect on the pyrexia, it cannot be said that horse-serum, whether antiviral or normal, modified the general course of the infection if administered when the temperature had already risen. That three of the animals should die with acute hæmorrhagic lesions recalling those recorded in a previous series (Ledingham and Barratt, 1929), while six should make good recoveries, would indicate a considerable variation in the pulmonary resistance of rabbits to intravenously inoculated virus.

It may be added that three of the surviving animals (A, E and G) were killed on the 28th day. A showed a very few residual pocks on the pulmonary pleura, E showed a very great many, while G showed none.

Histological examination showed healing necroses in the virgin uteri of all three, in the adrenals of E and in the ovaries of G. The other three survivors were tested for immunity on the 29th day by intravenous injection of 3 c.c. of the same batch of virus. Temperatures were daily recorded for a week, but no disturbance occurred. They remained well.

Brief Discussion on Data in Section II.

The experiments here recorded support the findings of others, that owing to the rapid union of vaccine virus with susceptible rabbit tissue, administration of antiviral serum must be prompt if complete neutralization is to be secured. We do not, however, take the view that this experimentally elicited fact renders antivaccinial serum therapeutically valueless. The justification for therapeutic use of antibacterial sera (*e. g.* antipneumococcal and antistreptococcal sera) is based experimentally on what are essentially prophylactic tests, for the obvious reason that delayed intervention in rapidly progressing septicæmias in small animals is usually ineffective in controlling the progress of the infection. The therapeutic method of test deserves, however, further investigation from the experimental point of view, and it may be that even in small animals, by correct adjustment of test dose, therapeutic effects may be elicited by delayed serum administration. In any case the complete effectiveness of antivaccinial serum as a prophylactic agent in experimental infections of small animals is in

our opinion sufficient justification for its therapeutic exploitation in similar but naturally acquired infections in man.

SUMMARY AND CONCLUSIONS.

(1) An antivaccinial serum has been secured by hyper-immunization of the horse with the virus of vaccinia.

(2) The antiviral body is found to be associated with the euglobulin as well as with the pseudo-globulin fractions of the serum, but not with the albumin.

(3) The euglobulin fraction contains the antibody in highest concentration per gramme of protein, but its absolute amount falls below that present in the pseudo-globulin fraction.

(4) In the course of hyperimmunization pursued intermittently over a period of nine months, the natural serum of the horse exhibited variations in potency, but it was established that falls in titre could be succeeded by maximal titres in response to further stimulation.

(5) The serum when administered simultaneously with the virus has proved highly effective in controlling experimental infections of rabbits with strains of virus of cutaneous and testicular origin.

(6) Though therapeutic effects from late administration of serum have not been definitely elicited in the experiments adduced, it is held that success in this direction may depend largely on dose of virus, and that further experimentation on therapeutic lines is desirable, if only to secure some valid basis for its exploitation in human infections with this virus.

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ADSORPTION EXPERIMENTS WITH THE ROUS SARCOMA VIRUS.

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THE results of different investigations on the adsorption of the virus of the Rous sarcoma have been variable. Fränkel and Mislowitzer (1930), Maschmann (1931), and Lewis and Mendolsohn (1931) found that it was adsorbed on aluminium hydroxide gels, but on the other hand Murphy (1931) states that the virus itself is not adsorbed by alumina, whereas the inhibitory substance, associated with the virus in the tumour filtrate, is so adsorbed. The type of gel used by Murphy was Willstatter's Cγ preparation. This was also the gel used by Maschmann in his experiments. Again Lewis and Mendolsohn consider that neither kaolin nor charcoal adsorb the virus while Maschmann found that both these substances could be used as adsorbents. The differences between these results are probably due to the different character of the adsorbents and the different amounts of them that were used ; my own results show that this last point is of great importance in the adsorption process. Consequently, in spite of the results which have already been published, it seems worth while to describe briefly the details of methods of purification of the Rous virus which yield preparations which are active in very small doses and which contain relatively only small amounts of impurities. The original suspension of the virus has been prepared by grinding Rous sarcoma No. 1 with sand, suspending this mash in Ringers solution to make a 4 per cent. suspension of tumour, spinning until the extract is fairly clear and then filtering through a paper pulp and sand-filter (Gye and Andrewes, 1926) under gravity. Maschmann, Fränkel and Lewis's results showing that the virus is adsorbed by alumina Cγ have been confirmed and also the eluting action of alkaline phosphates. It was found that di-sodium phosphate at pH 9·2 between the concentrations $\frac{M}{7}$ and $\frac{M}{100}$ easily removed the virus from the gel.

The effect of pH on the adsorption by alumina was first investigated since it was hoped that an optimum might be found as is usually the case in the adsorption of enzymes. A wide range of pH could not be investigated as the virus is unstable below pH 4·0 (Lewis and Michaelis, 1929). Equal volumes of filtrate were brought to pH 9·0, pH 7·0, and pH 5·0 with NaOH and HCl respectively, then equal quantities of alumina or kaolin were added and the pH again adjusted if necessary. After half an

hour's adsorption at room temperature the precipitates were spun off, washed on the centrifuge with water and then eluted with a volume of phosphate equal to that of the original filtrate. In some cases the residue from the first adsorption was readsorbed and the precipitates eluted together. It was usually found that adsorption was not complete at any pH after two treatments with 3 per cent. alumina Cγ, but that, testing both the residue and the elute, adsorption occurred equally well at pH 7·0 and pH 9·0. At pH 5·0 both the residue and the elute were less active than the solutions at a higher pH. Probably partial inactivation occurred. Table I gives the results of a typical experiment. The pH's given are those at which adsorption took place. The tumours produced by the residues were similar in size.

TABLE I.

No. of bird.	pH 9·0 elute.	pH 7·0 elute.	pH 5·0 elute.	Dose.	Remarks.
893	..	×××	××	0·5 c.c.	Filtrate adsorbed
901	××	..	(×)	0·5 ,,	0·3% Al Cγ once.
904	×××	×××	..	0·5 ,,	Eluted with M/7
895	..	××	×××	0·2 ,,	Na_2HPO_4 at pH 9·2.
903	×××	..	×	0·2 ,,	..
899	×××	×××	..	0·2 ,,	..

In this and all other tables the size of tumour at the death of the bird is indicated by the number of crosses. Four crosses are the maximum; one cross in brackets indicates a tumour no bigger than a pea. All birds were killed one month after inoculation unless it was necessary to do so earlier.

The effect of different quantities of alumina on the adsorption was then studied. Glenny (1931) found that the precipitation of diphtheria toxoid by potash alum was very greatly influenced by the quantity of alum used. Between 1 per cent. and 5 per cent. alum there was a maximum, whereas between 5 per cent. and 10 per cent. alum, the precipitation fell off, although the purity of the precipitate increased, as judged by the ratio of Lf units to mgm. N in the precipitate. The adsorption of the Rous virus is also influenced by the weight of gel used. Table II shows that with increasing concentration of alumina the virus is irreversibly attached to the gel. The volume of phosphate used for elution was in each case equal to the volume of the original filtrate.

TABLE II.

No. of bird.	0·15% alumina.		0·3% alumina.		0·6% alumina		0·9% alumina		Number of adsorptions.	Dose.
	Elute.	Residue.	Elute.	Residue.	Elute.	Residue.	Elute.	Residue.		
862	××××	..	××××	..	××	..	×	..	2	0·5 c.c.
860	×××	..	×××	..	×××	..	×	..	2	0·5 ,,
857	×××	..	××	..	×××	..	×	..	2	0·2 ,,
861	×××	..	××××	..	××	..	×	..	2	0·2 ,,
106	..	××	..	neg.	..	neg.	..	neg.	1	0·5 ,,
10	..	××××	..	××	..	×××	..	××	1	0·5 ,,
107	..	×××	..	××	..	×××	..	×	1	0·5 ,,
113	..	××××	..	××××	..	××	..	×	1	0·5 ,,

Maschmann (1931) found that it was possible to precipitate the phosphate from an alumina elute and to leave the activity in the supernatant fluid. This result has been confirmed. Calcium chloride was used as a precipitant and dilute soda was added to maintain a neutral reaction.

Kaolin between 0·5 per cent. and 2 per cent. was also found to adsorb the virus. This also took place most readily at a neutral reaction as the virus was slightly inactivated at pH 5·0. The virus was eluted with alkaline phosphate. The resulting solutions were not found to be so regularly active as those obtained by alumina adsorption and consequently more work on the purification of virus has been done with alumina. Lewis and Mendolsohn considered that kaolin did not adsorb the virus, since after grinding a filtrate with 25 per cent. kaolin they found that the residue was active, although it was water clear and gave no biuret reaction. The doses given were 5 or 10 c.c. On the other hand I have found that the elute from a kaolin adsorption was active in 1/20 c.c. and that the nitrogen of the infective dose was 0·00023 mgm. It is therefore evident that differences in amount of adsorbent used make a great deal of difference in the results. The large doses given by Lewis and Mendolsohn may have been necessary owing to the age of the fowls used by them. In full grown fowls larger doses are in general necessary to ensure the growth of a tumour. In all my experiments chickens of between 2 and 3 months of age have been used.

Since suspensions of virus containing relatively few impurities could be prepared by these methods, I thought that possibly the stability of such solutions might be either greater or less than that of the crude filtrates. Gye and Purdy (1930) showed that although incubation at 37° C. for 24 hours destroyed the activity of the filtrate, the addition of 1/10,000 HCN entirely inhibited the destruction during incubation for two days ; Mueller (1928) showed that the same was true for cysteine. Pirie and Holmes (1931) concluded that the loss in activity was due to an oxidation, not to a proteolytic change. Since free oxygen is not necessary for the oxidation to take place, both the substance oxidized and the substance which oxidizes it, either by removal of hydrogen atoms or the addition of oxygen, must be present within the tumour filtrate. Therefore, if by adsorption the virus were separated from any part of the inactivating mechanism, either the catalyst or the oxidizer, then, during incubation, the stability of the elute might be greater than that of the non-adsorbed filtrate and the addition of HCN should have no effect. For incubation at 37° C. sterile solutions were necessary, so preliminary experiments on the filtration of the elutes were carried out. I found that at pH 9·2 and at pH 7·4 the elutes could be filtered through a Berkefeld candle and still retain some of their activity. Comparisons of the activity of an elute before and after candling were made and showed that at both pH's a loss in activity occurred. Sterile filtrates of alumina elutes were then incubated at 37° C. with and without 1/10,000 HCN, and after 24 hours their activity was tested in chicks. Table III shows that inactivation proceeds in the same way as in the crude filtrate. Although this does not prove that the virus itself contains all parts of the oxidizing mechanism, but only that all parts have the same adsorptive behaviour as the virus, it does perhaps suggest that the complete system is closely attached to it.

TABLE III.

No. of bird.	0 hrs. at 37° C. elute.	24 hours at 37° C. Elute.	24 hours at 37° C. Elute with HCN.	24 hours at 37° C. Elute with cysteine.	Type of elute.
154	××××	(×)	×××	××××	Phosphate elute from kaolin.
153	×××	(×)	××	××××	Phosphate elute from kaolin.
929	××	neg.	×	..	Phosphate elute from alumina.
930	×××	neg.	neg.	..	Phosphate elute from alumina.

Since the stability of the fresh elutes was no greater than that of the fresh filtrates, their stability was then tested after drying. Five c.c. were pipetted into small Petri dishes and dried in vacuum desiccators over phosphorus pentoxide. The elutes retained their activity after drying for at least two months if they were frozen during the evacuation of the desiccator. If freezing did not occur the elute always lost its activity.

TABLE IV.

Nos. of birds.	Size of tumour.	Time dried.	Dose.	Type of solution dried.
334 and 335	neg.	12 days	1 c.c.	Kaolin precipitate.
334 and 335	neg.	12 ,,	1 ,,	NH_3 elute from kaolin.
577 and 578	,,	1 month	1 ,,	Na_2HPO_4 elute from alumina.
54 and 92	large	3 days	0·5 ,,	Na_2HPO_4 elute from kaolin.
54 and 92	,,	3 ,,	0·5 ,,	NH_3 elute from kaolin.
49 and 99	,,	2 ,,	1 ,,	Na_2HPO_4 elute from alumina.
133 and 135	,,	1 month	1 ,,	Na_2HPO_4 elute from alumina.
746 and 748	,,	2 months	1 ,,	Na_2HPO_4 elute from kaolin.

The biuret, ninhydrin, and Molisch reactions of these preparations of virus were frequently negative, so a study of the nitrogen contents of minimal infective doses was made. Table V gives the results obtained by different methods of adsorption.

Fifteen c.c. were usually taken for the estimation of ammonia. The solution was incinerated in a small Kjeldahl flask and then transferred to a distillation apparatus and the ammonia estimated by Nessler's reagent. Watchorn and Holmes (1927) found that by this method 0·01 mgm. of ammonia could be estimated. Blank estimations were always run on the reagents. The figures for the nitrogen of the infective dose are sufficiently uniform to show that the virus only contains or is accompanied by very small amounts of nitrogen. Whether this is protein nitrogen or not cannot be determined.

Although the preparation of these less impure suspensions of a virus has no very great interest in itself, it is possible that the use of such suspensions may enable more definite results to be obtained on the factors necessary for the growth or metabolism of the virus. The reactions by which viruses gain their energy are at present totally unknown, and until it is possible to cultivate them on synthetic media the most hopeful way of study seems to be that of

TABLE V.

Method of preparation of solution.	Active dose.	Nitrogen of dose.
Residue from kaolin adsorption adsorbed on alumina. Phosphate elute from this adsorbed on kaolin. Residue from this adsorbed on alumina at pH 6·0 and eluted with phosphate at pH 9·2. 0·3% alumina Cγ and 2% kaolin used	0·05 c.c.	0·0004 mgm.
Residue from adsorption with 0·3% Cγ alumina at pH 9·0	0·2 ,,	0·002 ,,
Filtrate adsorbed with Merck's animal charcoal. Residue adsorbed 0·3% alumina three times. Gel eluted with Na_2HPO_4. Elute candled through Berkefeld. Alumina Cγ used.		
Elute before candling	0·2 ,,	0·001 ,,
,, after ,,	0·5 ,,	0·008 ,,
Residue from charcoal	0·5 ,,	0·002 ,,
Filtrate adsorbed 2% kaolin. The residue adsorbed 0·3% alumina at pH 6·0. Gel eluted with Na_2HPO_4. The elute was candled at pH 7·6.		
Elute after candling	0·2 ,,	0·0003 ,,
Residue after candling	1·0 ,,	gave no tumour
Filtrate adsorbed with 0·1% alumina until free from hæmoglobin. Residue re-adsorbed on 0·3% alumina Cγ three times at pH 6·5 and eluted with Na_2HPO_4 at pH 9·2		
Elute	0·1 ,,	0·001 mgm.
Filtrate adsorbed with kaolin at pH 6·0. Kaolin eluted with phosphate at pH 9·2		
Elute	0·05 ,,	0·0002 ,,

as pure a preparation of virus as it is possible to obtain. Such preparations may only contain dying virus, but just as a study of dead bacteria has brought to light a great number of facts about the metabolism of living bacteria so a dying virus may furnish some clues to the life of an active one.

SUMMARY.

The adsorption of the Rous sarcoma virus onto, and the elution of it from, alumina and kaolin is described.

Such elutes are inactivated during incubation as rapidly as the crude filtrates, and are preserved by cyanic acid in the same way.

The nitrogen of the infective dose of the elutes is of the order of 10^{-3} mgm.

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EXPERIMENTS ON THE PRODUCTION OF TUMOURS ON THE SOMATIC MUTATION HYPOTHESIS.

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THE somatic mutation hypothesis postulates that tumours are the result of mutations of somatic cells. It follows, that agents known to produce genetic mutations, and other derangements in the genes of germ-cells, will be chosen to put this hypothesis to the test of experiment. It is now a well-established fact that X-rays and the β- and γ-rays from radium are powerful agents for the production of genetic mutations. Furthermore, exposure to these radiations has often been followed by the occurrence of cancer. For these reasons radiation has been chosen to test the hypothesis. I have shown (1928, 1930) that high concentrations of carbon dioxide give rise to derangements of chromosomes, and to the production of non-disjunction in Drosophila. I have therefore used high concentrations of CO_2 in addition to β-radiation in this investigation.

The following is a summary of the experiments carried out:

(1) *Experiments with testis.*—The testes of rats from two to four weeks old were removed, minced, and divided into two portions. One portion was exposed to a sublethal ($\frac{1}{4}$, $\frac{1}{2}$ and $\frac{3}{4}$) dose of radiation, and then a small quantity inoculated in the right thigh muscles and right flank of the castrated animals, and also into brothers and sisters of the castrated animals. The other non-radiated portion of the emulsion was similarly inoculated into the left side.

Thirty-one animals were thus treated. In one case, described later, a tumour resulted from the radiated material.

(2) *Experiments with kidney.*—(*a*) One kidney was removed and cut into small pieces (1 mm.^{3}). Some pieces were exposed to a sublethal dose of β-radiation; the others served as controls. These were inoculated under the skin of the rat, six pieces into each rat. Eight rats were used and no tumours resulted.

(*b*) *In vitro* cultures of rat kidney were kept in from 70–150 mm. of CO_2. Cultures in air served as controls. When good "growth" was observed, they were inoculated into rats. Fifty-nine cultures were used, and no tumours resulted.

(3) *Experiments with spleen.*—The spleen was removed and treated as in the kidney (*a*) experiments. Thirty-five rats were used, with negative results.

(4) *Experiments with embryo skin.*—(*a*) A strain of inbred rats was used so as to avoid foreign tissue reactions. Two-week-old embryos were taken, their skins removed and minced; a portion was given a sublethal dose of β-radiation, and the remainder served as a control. Small quantities were inoculated into the right (radiated) and left (control) flanks of normal rats. In many cases dermoid tumours containing hair as large as $3 \times 1{\cdot}5$ cm. resulted, but no differences between experiment and control were observed. Twenty-six animals were thus treated.

(*b*) In another series a pregnant rat and mouse were radiated over the abdomen. Subsequently the embryos were removed, and their skins used for inoculation. Fourteen rats and five mice were inoculated. Only dermoid cysts containing hair resulted.

(*c*) Embryo skin emulsion was kept in pure CO_2 for 4 hours, and then inoculated into suitable controls. Fourteen rats were inoculated and no tumours resulted.

It is seen that in all these experiments only one tumour occurred. This had the following history: The testes of a male rat, 13 days old, were taken; they were minced with knives; half the emulsion was exposed for 15 minutes to radiation (applicator of 60 mgm. radium element, area 4 sq. cm., screen 0·12 mm. silver); about 3 cm. of the radiated emulsion was inoculated into the right thigh muscles and the right flank of a sister of the castrated rat, non-irradiated emulsion into the left thigh and flank; 72 days afterwards the animal was killed. No tumour was felt ante-mortem, but post-mortem two nodules joined together, each measuring $10 \times 5 \times 5$ mm., were found embracing the upper end of the right femur. The tumour was dissected away from the surrounding normal tissues with little difficulty; it presented on section a smooth white surface and was of the consistency of firm jelly; there were no necrotic areas. Histologically the tumour was surrounded by a narrow layer of fibrillar connective tissue separating it from the normal tissues, such as muscle and adipose tissue, which were not invaded, only displaced. This connective tissue was in many places densely infiltrated with lymphocytes and plasma-cells, and occasionally these cells were present also in the superficial layers of the tumour. For the most part the tumour consisted of healthy cells presenting numerous mitoses; areas of early degeneration were also present where there was vacuolation of the cytoplasm and

pyknosis, and loss of structure of the nuclei. Choosing a place where mitotic figures were numerous, the tissue was found to be composed of cells of almost entirely one kind apart from an occasional lymphocyte. These cells had a finely fibrillar or granular cytoplasm, which presented some vacuolation; cell margins were difficult to define and the tissue had the appearance of a syncytium; the nuclei were large and oval, measuring often 1/60 × 1/120 mm.; they stained palely, were firmly granular and contained one or more small nucleoli. These cells are shown in Fig. 1 (A). Sometimes, as in Fig. 1 (B), the nuclei were spindle-shaped and lay in bundles, many pointing in the same direction.

In several parts of the tumour remains of seminiferous tubules were seen, consisting externally of flattened connective-tissue cells, and several layers of

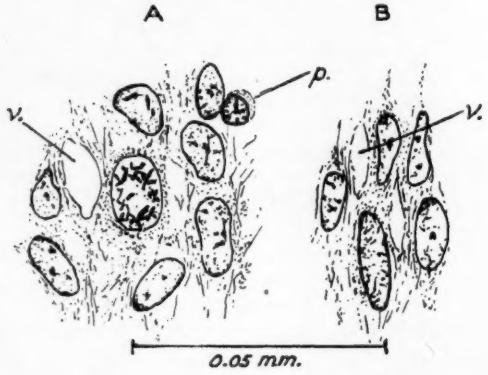

FIG. 1.

p., lymphocyte; *v.*, vacuole in cytoplasm.

spherical cells bounding a central cavity; the round cells were not dividing and there was no evidence of spermatogenesis. In some cases the tubules consisted of only flattened connective-tissue cells enclosing a cavity containing cell-*débris;* in other cases the flattened connective-tissue cells were absent and the spermatogonia had spread among the tumour-cells for a short distance. Sometimes the central cavity was absent, and the spermatogonia formed a solid cell mass (as shown in Fig. 2). Only two mitoses of the spermatogonia cells were seen.

The conclusion is that the tumour is a sarcoma; and from the fact that it occurred at the site of inoculation, and contained remains of seminiferous

FIG. 2.—Microphotograph, × 225, showing remains of seminiferous tubule. On the left side the tumour is degenerated; healthy tumour-cells are to be seen on the right of the tubule.

FIGS. 3 and 4.—Microphotograph, × 225.

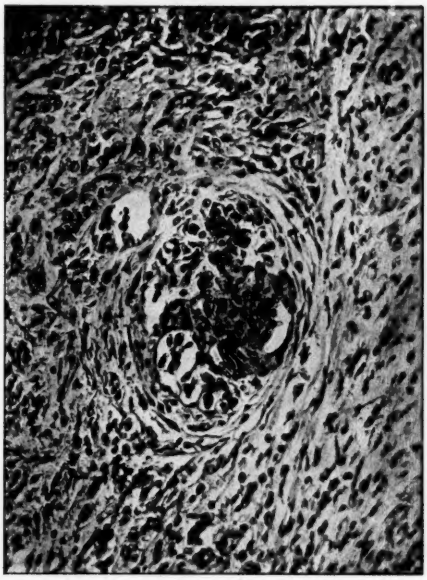

FIG. 2.

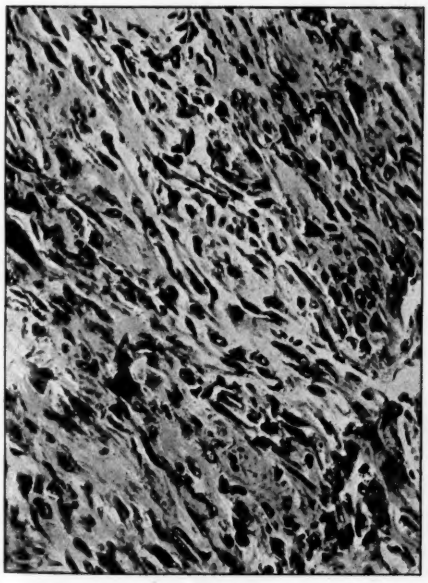

FIG. 3.

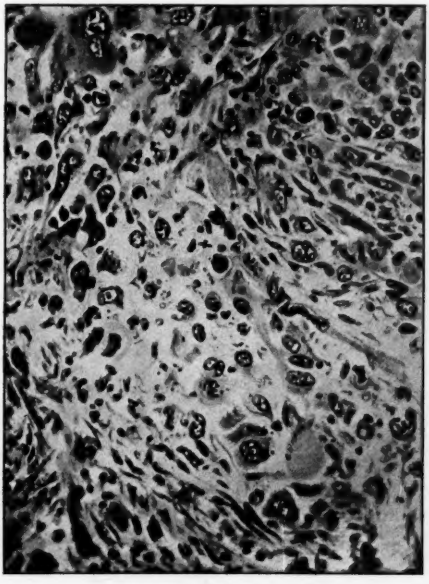

FIG. 4.

tubules, it is inferred that it was derived from the inoculated material. The tumour grew like an inoculated tumour, pushing the normal tissues aside, was easily separated by dissection, and did not infiltrate; nevertheless, the possibility that it was derived from connective-tissue cells of the inoculated rat cannot be entirely ruled out. Small pieces of the tumour were placed in both flanks of twenty-four rats, with negative results.

DISCUSSION.

Since the original pronouncement by Muller (1927) that X-rays greatly increase the production of mutations, a considerable literature has already arisen. Furthermore, somatic mutations have been produced by X-rays by Spencer (1928), Patterson (1928), and Timofeeff-Ressovsky (1928). Many lines of discussion are thus opened up; here the only one to be followed is the consideration of the chance of producing a tumour in the way which has been tried. Although the chance of producing a mutation is greatly increased by X-rays—15,000 per cent. greater in some of Muller's experiments—nevertheless the normal rate of occurrence is so small that probably many thousand cells require to be radiated in order to produce a single mutation. Moreover, it is not until a cell has divided that a mutation can become manifest. It follows that when germ-cells or the somatic cells of developing embryos are used for the investigation of mutations, there is a good chance of detecting any mutation which may be produced. Where, however, an emulsion of cells is inoculated into animals, only a thin layer of cells at the surface remains viable; the whole of the central mass of cells degenerates and dies, since they are out of contact with the blood-supply.

Furthermore, supposing that one of the viable cells at the surface undergoes mutation, it by no means follows that the mutation will be of such a nature as to produce a tumour; mutations will affect cells in very many ways, of which power to grow either into a simple tumour or a cancer is only one. It follows that the chance of producing a tumour by radiating an emulsion of cells, and then inoculating them, is very much smaller than the chance of producing recognizable mutations in germ-cells or in somatic cells radiated *in vivo*.

Considering the production of cancer by X-rays or radium, and still assuming the somatic mutation hypothesis, it is here also evident that a mutation to produce a tumour must be a very rare event. It is necessary to expose the skin and subcutaneous tissues to X-radiation repeatedly over periods of months before a tumour results (see "Appendix"). Here millions of cells are repeatedly exposed, any one of which is in a position to suffer a mutation and give rise to a tumour. It is possible that a single exposure might be sufficient, just as a single exposure to tar is said occasionally to be followed by cancer. It is not therefore to be wondered at that in this investigation only a single tumour has resulted. Nevertheless, by so much is its importance magnified. Prolonged irritation, prolonged inflammatory reaction, sepsis, necrosis and such possible factors in the production of cancer have been ruled out in these experiments. Instead has been substituted a treatment known to produce mutations. Furthermore, changes in the host have been largely ruled out since the radiation

was applied to the cell *in vitro* before inoculation. Even if oft-repeated, this result will probably not prove beyond all doubt that cancer is brought about by the mutation of a somatic cell, apart from the possibility that the tumour was derived from fibroblasts of the host ; but it might lead to the ideal experiment in which a single cell was taken, radiated and inoculated and gave rise to a cancer.

CONCLUSION.

A sarcoma-like tumour appeared in a rat at the site of inoculation of a testicular emulsion which had been exposed to radiation *in vitro*. This was the only tumour arising from many experiments of a similar kind. This work was carried out under a grant made by the British Empire Cancer Campaign. The radium used was on loan from the Medical Research Council.

APPENDIX.

In order to illustrate the conditions under which radiation will give rise to tumours by means of *in vivo* exposures, a brief description is given of two sarcomas thus produced. Eight rats were taken ; three died after three months' radiation ; of the remaining five, two developed sarcomas ; the remaining three died after a year and presented no tumours.

The first tumour-bearing animal was given at 0 days 45 minutes' radiation from the applicator described on p. 379 to the skin of the back ; from 21–28 days the area was given 1 minute three times a week ; 28–42 days 3 minutes three times a week ; 42–45 days, 5 minutes; 45–50 days, 7 minutes; 50–235 days, 10 minutes three times a week ; 235th day an exposure of 35 minutes ; 251–266 days, 10 minutes three times a week ; 266–416 days, 5 minutes three times a week ; on 380th day a tumour appeared at the site of exposure and radiation was stopped, and on the 416th day the tumour measured 32 × 21 mm. The tumour was removed by operation and inoculated into twenty-four rats. Two tumours resulted, of which one was absorbed. The other was inoculated into twenty-four rats and no tumours resulted. The photograph (Fig. 3) is of the original tumour, which was a fibrosarcoma.

The second tumour-bearing animal was given at 0 days 45 minutes, and subsequently the same treatment as the former animal, except that it did not have any radiation on the 235th day. A tumour appeared on the 257th day and was removed and auto-grafted. It recurred at the original site, and on the 415th day measured 18 × 18 mm. It was removed and inoculated into twenty rats. Five tumours resulted. Subsequently it was carried through twenty-one generations, when it was allowed to die out. The photograph (Fig. 4) is of a tumour of the twenty-first generation ; the tumour was a sarcoma. Many of the sarcoma-cells were multi-nucleated and giant sarcoma cells were present. These two animals were sent to Dr. Murray at the Imperial Cancer Research Fund, and I am much indebted to him for the carrying out of the investigation by inoculation of these tumours.

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AN INVESTIGATION INTO THE STREPTOCOCCI FOUND IN CERTAIN DISEASES OF THE SKIN.

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THE *rôle* of the streptococcus in the production of diseases of the skin is a subject which still leads to considerable difference of opinion, and it seems that until the bacteriology of these diseases has been more fully worked out no satisfactory conclusion can be reached. In spite of the amount of work which has been done on this subject little has been achieved in the identification of the streptococcus responsible.

Erysipelas and scarlatina, though both are due to streptococci and both give rise to skin-lesions, have been excluded from the scope of this investigation.

Impetigo, as described by Tilbury Fox (1864), is now generally recognized as streptococcal in origin since the work of Balzar and Griffon (1897) and Sabouraud (1900). The impetigo of Bockhart (1887) is a folliculitis, staphylococcal in origin, and in this paper the term " impetigo " refers to the former variety only.

Sabouraud (1904) also drew attention to the fact that the streptococcus may be the cause of pityriasis and of intertrigo. This streptococcal intertrigo is chiefly found behind the ear, but may also be found in other folds, such as the conjunctival and the naso-labial folds. Sabouraud has also (1927) described the extension of these intertrigos as an eczematoid condition of the neighbouring skin, particularly of the scalp and face. This, he states, is also streptococcal in origin. Haxthausen (1927, *a*) confirms Sabouraud's findings in impetigo, and describes the pityriasis caused by streptococci, which he calls " pityriasis streptogenes." He also draws attention to a form of dermatitis of the hands, closely resembling mycotic dermatitis, which he claims to be streptococcal. Sabouraud (1928) has published a very full account of these diseases.

Method of Isolating Streptococci from Skin-Lesions.

The chief difficulty in isolating streptococci from the skin is the presence of other organisms, chiefly staphylococci, which overgrow the streptococci. To obviate this Sabouraud used the method of Balzar and Griffon (1897). The serum from an impetigo vesicle is drawn up the stem of a Pasteur pipette half full of serum broth. The end of the pipette is then sealed, and it is incubated for nine or ten hours at 39° C. The contents of the pipette are then examined microscopically, and owing to the more rapid growth of streptococci in anaërobic medium than of staphylococci, their presence is easily detected. Haxthausen (1927, *a* and *b*) has evolved a method, by adding a solution of crystal violet to broth or blood-agar plates, by means of which the growth of streptococci is not interfered with while that of staphylococci is inhibited.

These authors claim that by these methods streptococci can be found in practically 100 per cent. of the lesions described above.

If some such method is not employed there is no doubt that the presence of streptococci can easily be missed owing to the overgrowth of other organisms. Balmain (1926) found streptococci in only 43 out of 66 impetigo lesions examined from 56 cases. His method was to streak blood-plates with serum from the vesicle or from under the crust.

None of these writers has made any serious attempt to classify the types of streptococci found. Sabouraud contents himself with the recognition of microscopical characters ; Haxthausen (1927, *a*) states that " the streptococcus is hæmolytic in most cases." Balmain (1926) states that in his investigation no attempt was made to differentiate the types of streptococci, but he mentions that the streptococcus was hæmolytic in 25 cases out of 43. Flehme (1920), however, examined 55 cases of impetigo, and found in every case *S. longus* producing hæmolysis, and acid in mannite-litmus-agar.

In the present investigation the crystal violet method of Haxthausen was used to isolate the streptococci. Since the material for investigation was obtained from patients at some distance from the laboratory the use of solid medium was not practicable ; scales, crusts and serum were inoculated directly from the patients into tubes containing 2 c.c. of 1 per cent. glucose broth of pH 7·5 ; these were conveyed to the laboratory, where sufficient stock solution of crystal violet was added to make the concentration required. A concentration of 1 in 500,000 was found to be best, but this is not very critical. This crystal violet solution keeps indefinitely, but if mixed with broth it is apt to be reduced to its leuco base, especially if incubated at 37° C., which invariably made the broth colourless in 24 hours if no bacterial growth took place. Where growth did take place the broth might remain coloured for several days.

After 24 hours' incubation at 37° C. the broth was examined microscopically and the results noted. The findings are given in Table I, and show that out of 52 cases of the streptococcal diseases, as defined by Sabouraud, streptococci were seen in 51, whereas out of 16 non-streptococcal dermatoses streptococci were seen only in two cases, both of them rosacea.

The present investigation thus bears out the work of Sabouraud that streptococci are almost invariably present in impetigo, and in certain well-defined forms of intertrigo, pityriasis and dermatitis.

TABLE I.—*Micro-organisms Seen in Broth Culture of Crusts, etc., from Cases of Streptococcal (Nos. 1–4) and Non-streptococcal (Nos. 5–11) Dermatoses.*

	Total examined.	Micro-organisms seen.		
		Streptococci.	Staphylococci.	Bacilli.
1. Impetigo	24	23	11	2
2. Retroauricular intertrigo	16	16	9	7
3. Pityriasis streptogenes	7	7	7	1
4. Streptococcal dermatitis	5	5	4	0
5. "Seborrhœic" intertrigo	1	0	0	0
6. "Seborrhœic" pityriasis	2	0	1	1
7. "Eczema"	2	0	0	0
8. Psoriasis	2	0	2	0
9. Sycosis	1	0	0	0
10. Erythematosus	2	0	2	0
11. Rosacea	4	2	4	0

The isolation of the streptococci from these specimens was not invariably successful. In the first place, subcultures must be made from the broth in 24 hours at the latest or they will be unsuccessful. Bacilli, especially of the *proteus* type, spoilt several plates by overgrowing other organisms until the method of Browning and Gulbrausen (1918) of adding thallium nitrate to the culture media was adopted. This was found to be entirely effective. When, therefore, bacilli were seen in the 24-hour broth culture a subculture was made into broth containing 0·06 per cent. thallium nitrate as well as crystal violet. No trouble was experienced on subsequent plating. Stronger concentrations of the thallium nitrate were found to inhibit the growth of streptococci and staphylococci. The addition of thallium nitrate to blood-plates was found to be equally effective in inhibiting bacillary growth, but it also interfered with hæmolysis, causing hæmolytic streptococci to appear non-hæmolytic; though subculture of these non-hæmolytic colonies on to non-thallium plates showed a return of hæmolytic power.

Agar plates containing 5 per cent. human blood were then lightly inoculated from the broth and spread. After 24 hours' incubation at 37° C. the plates were examined and the various types of streptococci provisionally identified.

Individual colonies of streptococci were then picked off and inoculated on agar slopes, which were examined for purity after 24 hours' incubation at 37° C. From these stab cultures were made into semisolid agar, which formed the stock cultures. These were kept in the 37° C. incubator and subcultured every three or four weeks. Blood plates were inoculated from these stock cultures at intervals, and no noticeable variation in the behaviour of the streptococci was observed even after a lapse of several months.

Types of Streptococci Encountered.

A streptococcus frequently found in the present investigation showed small translucent colonies on human blood-agar plates, and did not perceptibly

affect the surrounding medium either by decolorizing it or producing a green discolorization. The size of these colonies and their translucency renders them easily missed, especially if the plate is examined from the back, though a careful examination of the surface of the medium shows a profuse growth of these organisms. If other organisms are present their presence is more easily overlooked. This seems to be the same organism as that described by Mandlebaum (1907–1908) as *S. saprophyticus*, by Zangemeister (1910) as *S. anhæmolyticus*, and found by Rosenow (1914) among streptococci from joint fluid in cases of rheumatism.

Examples of that heterogeneous collection, the *viridans* group, were encountered, but so occasionally that their presence was evidently accidental. *S. viridans* was never found without either *S. hæmolyticus* or *S. anhæmolyticus*.

In the present series in twelve cases of impetigo streptococci were successfully isolated and a hæmolysis test performed.* A streptococcus definitely hæmolytic in character was found in every case. Table II shows the results in a few cases, including those with the lowest and highest hæmolysin production. The streptococci isolated from two of the cases of rosacea were also *S. hæmolyticus*, but this streptococcus was isolated from no other dermatosis, whether presumably due to streptococci or not.

TABLE II.—*Results Read after Two Hours at 37° C. A Final Dilution of* 1 in 8 *Corresponds Roughly with Cumming's Requirements.*

Final dilutions of 24-hour serum broth-culture in 3 per cent. rabbit-cell suspension.	1 in—					
	2.	4.	8.	16.	32.	64.
Stock *S. hæmolyticus* (surgical)	C.L.	C.L.	C.L.	C.L.	A.C.L.	P.L.
P52a. *S. hæmolyticus*	,,	,,	,,	,,	C.L.	C.L.
P51a. ,,	,,	,,	,,	,,	A.C.L.	P.L.
P62. ,,	,,	,,	,,	A.C.L.	P.L.	,,
P68. ,,	,,	,,	,,	C.L.	T.L.	N.L.
P1a. *S. viridans*	N.L.	N.L.	N.L.	N.L.	N.L.	,,
P70b. *S. anhæmolyticus*	T.L.	,,	,,	,,	,,	,,

C.L. = complete lysis, A.C.L. = almost complete lysis, P.L. = partial lysis, T.L. = trace of lysis, N.L. = no lysis.

In 15 cases of intertrigo affecting the retro-auricular fold a streptococcus was successfully isolated. In each case this streptococcus had the characters of *S. amhæmolyticus*. In the primary subculture on human blood plate a faint decolorization of the medium immediately surrounding the colony was occasionally observed, but this disappeared in subsequent subcultures.

This retro-auricular intertrigo frequently spreads on to the scalp and face,

* For hæmolytic streptococci the standard the writer has adopted is that used by Cumming (1927), and a streptococcus was called hæmolytic only if 0·2 c.c. or less of a 24-hour serum broth culture completely hæmolysed 1 c.c. of a 3 per cent. suspension of washed rabbit cells kept at 37° C. for two hours. This extended hæmolysis test provided a definite and easily applied standard for hæmloytic streptococci.

and, in very extensive cases, the body, especially the folds at the elbow-joints, axillæ, groins, behind the knees and the umbilicus, which may be the seat of a red, moist, scaly dermatitis. As this clears up it leaves a fine, branny scaliness —pityriasis streptogenes. Where this dermatitis existed scales from these lesions as well as from behind the ears were examined and in every case *S. anhæmolyticus* was found.

The incidence of this form of dermatitis without the infection of the retro-auricular fold seems much less common, but in five cases of this nature from which a streptococcus was successfully isolated, *S. anhæmolyticus* was found. One case affected the hand and the interdigital folds, one the nose only, one the face generally, one the scalp, and one the external auditory meatus.

Pityriasis streptogenes without other streptococcal lesions was not often found. Three cases came into this series, and in each case *S. anhæmolyticus* was isolated.

It is to be expected that impetigo and retro-auricular intertrigo might be found together. Two cases in this series showed both lesions at the same time, and in both cases *S. hæmolyticus* and *S. anhæmolyticus* were isolated.

Clinically this form of intertrigo with its associated dermatitis is often very chronic in its course. In the writer's experience it may last as long as several years. The usual duration even under constant treatment is several weeks. One patient, a boy, aged 4, had had an intertrigo and dermatitis of the scalp for two years previous to admission, and had been under active antiseptic treatment in the ward for two months before cultures were taken, yet *S. anhæmolyticus* was isolated without difficulty. As has been stated, the usual course is for a pityriasis to remain after the dermatitis has dried up.

Impetigo is a much more acute disease, and with appropriate treatment it ought to be cured in ten days. Sometimes it leaves behind a pityriasis, but not with the same regularity as occurs after streptococcal dermatitis. This can easily be explained if, as seems to be the case from the results of the present investigation, these two forms of infection are caused by different varieties of streptococci. From three cases of impetigo *S. anhæmolyticus* was isolated as well as *S. hæmolyticus*, though there was at the time no clinical evidence of the presence of the former organism. It is suggested that from such mixed infections arise the cases of pityriasis following impetigo.

S. viridans was isolated from three cases of impetigo along with *S. hæmolyticus*, and with *S. anhæmolyticus* from one case of retroauricular intertrigo. It seems, therefore, to have no part in the production of these diseases.

SUMMARY.

Following the crystal violet method of Haxthausen, the findings of Sabouraud and others of the presence of streptococci in cultures from the lesions of impetigo and certain forms of intertrigo, dermatitis and pityriasis (as defined by Sabouraud) is confirmed.

The streptococcus responsible for the production of impetigo seems to be *S. hæmolyticus*, and for the other diseases mentioned, *S. anhæmolyticus* of Zangemeister.

In conclusion the writer must express a deep debt of gratitude to Prof. Tulloch for his great help and supervision in the conduct of this investigation; to Dr. James Craigie for carrying out the animal experiments necessary and much other valuable help; and to the rest of the staff of the Bacteriological Laboratory of the University of St. Andrews for their assistance.

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THE SERUM TREATMENT OF ANAËROBIC (*VIBRION SEPTIQUE*) INFECTIONS IN MICE.

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ANTITOXIC sera have been used for several years in the prevention and treatment of anaërobic infections in man, but it has recently been suggested (Robertson and Felix, 1930) that it is no longer sufficient to rely solely on antitoxin, and that the ideal serum should contain antibacterial elements as well. These authors reported that an antibacterial serum which had been prepared by injecting washed and heated *Vibrion septique* bacilli into a horse, and which contained no "antitoxin," protected mice against the injection of washed spores to which calcium chloride had been added as activator.

We have tried to obtain an antibacterial serum in the horse by a similar method of immunization, using the same strain of *Vibrion septique* (Pasteur

III), and the same technique of testing against a spore-calcium mixture injected intramuscularly. According to Robertson and Felix, the preparation of an effective " O " immune serum containing no antitoxin necessitates a prolonged course of injections with very large quantities of washed and heated bacilli. When, after some months, the protective titre of our horse's serum was *nil*, it was thought that the heavy expense of further injections was hardly justifiable in view of the satisfactory results obtained with antitoxic sera in the same series of experiments. The immunization of the horse was accordingly discontinued. Unfortunately the horse immunized at the Lister Institute had died, apparently from protein shock, and, as its serum from an earlier bleeding has been almost entirely used up, it has not been possible to repeat the protection experiments of Robertson and Felix with antibacterial serum.

The investigation has been continued with antitoxic sera of varying potency, including G.G. 2639, the one used by Robertson and Felix, who found that it was able to protect only a small number of mice ; 20 per cent. of mice survived for 96 hours in one of their experiments, in which the serum was given intramuscularly in doses of 1·0 c.c. 48 hours before the spore-calcium mixture. Similar results were obtained by us ; 29 per cent. of 28 animals were protected, whereas untreated animals which received one-hundredth of the test dose died within 16–18 hours. A second serum protected 50 per cent. of 30 animals, but a third only 15 per cent. of 20 animals when 1·0 c.c. was injected, although 0·1 c.c. had protected 12 per cent. of 24 animals, and 0·5 c.c. 33 per cent. of 24 animals. In this instance 1·0 c.c. was clearly larger than the optimum protecting dose, some of the deaths at 1·0 c.c. being due possibly to some toxic effect of the serum. This led us to consider the advisability of giving smaller doses of serum than 1·0 c.c., and comparing the results obtained by different routes of injection.

In the following experiments (Table I) 0·5 c.c. of a serum of the same number of antitoxin units* (280 per c.c.) as G.G. 2639 was injected intramuscularly, intraperitoneally and intravenously, (*a*) at the same time as the spores and activator, and (*b*) five hours later. The serum was tested against at least 100 fatal doses of spores + activator.

TABLE I.—*Protection of Mice with* Vibrion septique *Antitoxic Serum Against* Vibrion septique *Spores + Calcium Chloride.*

Route of injection of serum (0·5 c.c.).	When serum injected.	Number of mice.	Percentage survived 96 hours.
(*a*) Intramuscular	Same time as spores + activator	24	17
Intraperitoneal		24	37
Intravenous		24	37
(*b*) Intramuscular	5 hours after spores + activator	12	0
Intraperitoneal		12	0
Intravenous		22	55

The infecting dose (0·7 c.c.), which was injected intramuscularly, consisted of spores, opacity II/100 (Brown's tubes), 0·25 c.c. + calcium chloride, 3 per cent. solution, 0·25 c.c. + saline, 0·2 c.c. Spores, opacity II/10,000, + activator, killed 100 per cent. of 12 mice within 16–18 hours.

* The unit quoted is the provisional unit of Glenny, Llewellyn-Jones and Mason (1931).

Absorption of serum was slowest by the intramuscular route, which in experiment (*a*) has given the poorest results. In experiment (*b*), as was expected, the serum was quite ineffective when given intramuscularly or intraperitoneally 5 hours after infection; when injected intravenously it saved 55 per cent. of mice—a striking result, as the animals were practically moribund at the time of injection.

In a subsequent experiment three doses of 0·25 c.c. of serum were given intravenously thrice, *i. e.* at the time of infection, 3 hours afterwards and 24 hours afterwards; 56 per cent. of 23 mice survived, whereas only 6 per cent. of a group of mice given 1·0 c.c. of serum intramuscularly, 48 hours before infection, lived.

Tests were also carried out with a concentrated and more potent serum, RX 418, to which no preservative had been added. The intravenous and intraperitoneal routes were selected for the injections of serum. The protocols of four experiments are given in Table II:

TABLE II.—*Protection with Concentrated Antitoxic Serum, RX 418 (1000 units per c.c.).*

	Total serum injected in c.c.	Doses and method of injection.	Number of mice injected.	Percentage survived 96 hours.
A.	0·8	0·4 ½ hour after infection intravenously	24	88
		0·2 24 hours after infection ,,		
		0·2 48 ,, ,, ,,		
B.	0·7	0·3 4 ,, ,, ,,	24	75
		0·2 24 ,, ,, ,,		
		0·2 48 ,, ,, ,,		
C.	1·3	0·5 48 hours before ,, intraperitoneally	24	100
		0·5 4 ,, ,, ,,		
		0·3 24 hours after ,, ,,		
D.	1·3	as above	29	92

In tests A and B, in which the concentrated serum was given intravenously after the infecting dose, 88 per cent. and 75 per cent. of mice survived. In experiments C and D, in which larger doses of serum were injected intraperitoneally before and after the infecting dose of spores + activator, 100 per cent. and 92 per cent. survivals were obtained. In view of the virulence of the infection in the absence of serum (in each experiment one-hundredth of the test dose killed all of 6 mice injected within 16–18 hours), these results are to be considered as eminently satisfactory.

DISCUSSION.

It is well known that *Vibrion septique* produces a powerful soluble toxin, very rapid in its action. When the toxin is allowed to act in small doses on an isolated organ such as rabbit's uterus, a spasmodic contraction of the muscle is caused within a few minutes, and there is a diminution of the spontaneous

contractions ; larger doses paralyse the muscle completely (Buttle and Trevan, 1928). We also know that necrosis is readily produced at the site of injection of toxin in the experimental mouse, guinea-pig or rabbit.

It seems probable, too, that the gangrene resulting from a natural infection is mainly the result of the toxin produced by rapidly proliferating bacteria. If, however, antitoxin is injected, the toxin which passes out from the local lesion is neutralized, and the animal survives. Extension of the local lesion is prevented.

In experimental infections with spores and activator in mice, local swellings and gangrene have been fairly common even in serum-treated animals which have survived, and have sometimes been followed by sloughing, but, as Robertson and Felix have pointed out, this is a very different type of infection from the fulminating gangrene which is the rule in untreated animals. Calcium chloride has of course a slight necrosing action, but the local gangrene in the treated mice is presumably mainly due to unneutralized toxin ; the antitoxin has been unable to penetrate from the general circulation into the damaged area locally.

Robertson and Felix do not underrate the value of highly potent antitoxic serum, and recognize that in neutralizing the toxin it deprives the bacilli of the means of attacking tissue beyond the area necrosed by the calcium, and in this way it also inhibits proliferation indirectly. They postulate, however, a further property on the part of the " O " immune serum, viz. that of inhibiting the multiplication of the bacilli so that they are unable to produce toxin and cause a generalized gangrene. In the absence of supplies of " O " serum we have been unable to do any experiments based on this hypothesis. Our main concern has been how far antitoxic serum would protect. As mentioned by Robertson and Felix, antitoxin given in advance does protect against culture of *Vibrion septique*. This observation has been made many times since anti-*Vibrion septique* serum was first made during the war. The experimental data given in Tables I and II show that antitoxic sera are also extremely effective against washed spores + calcium chloride. These results are at variance with those obtained by Robertson and Felix in only one respect, viz. the degree of protection obtained. More potent sera have been at our disposal, and we have also varied the route of administration. By repeated small doses of concentrated serum given intraperitoneally or intravenously we have been able to protect 75 to 100 per cent. of mice (Table II). Even with an unconcentrated serum of similar potency to the serum used by Robertson and Felix we have been able to overtake an infection of 5 hours' duration when the mice were practically moribund ; the serum had to be given intravenously, but 12 of 22 mice recovered (Table I).

The obvious indication from these experiments is that, when antitoxin is used therapeutically in the treatment of gas gangrene in man, it should be given intravenously in large doses and at the earliest possible moment.

SUMMARY.

1. *Vibrion septique* antitoxic serum confers complete protection against massive doses of living culture of *Vibrion septique*.

2. It is also highly effective in the prophylaxis and treatment of infections produced by washed spores with calcium chloride as activator.

3. A concentrated antitoxin, injected intravenously or intraperitoneally in divided doses, protected 75 to 100 per cent. of mice against 100 fatal doses of spore-activator mixture.

4. Mice nearly moribund 5 hours after infection recovered after the intravenous administration of antitoxin.

5. No experiments could be carried out with " O " immune serum similar to that described by Robertson and Felix.

6. A most important indication from this research is that, when antitoxin is used therapeutically in the treatment of anaërobic infections in man, it should be given without delay in large doses by the intravenous route.

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STUDIES ON PROTEIN-FREE SUSPENSIONS OF VIRUSES. III.—THE RESISTANCE TO HEAT AND DISINFECTANTS OF PROTEIN-FREE ELUATES OF A BACTERIOPHAGE AND FOWL-POX VIRUS.

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IN a previous communication (Kligler and Olitzki, 1931) a method was described which made it possible to obtain active, protein-free suspensions of bacteriophage and fowl-pox virus respectively. It was shown further (Kligler, Olitzki and Ashner, 1931) that such virus suspensions behave differently with regard to cataphoresis and resistance to acid from those in protein-containing substrates. In the present paper we shall present the results of observations on the relative resistance to heat and disinfectants of a coliphage and fowl-pox virus in protein and protein-free suspensions.

More precise knowledge of the resistance of viruses to heat, and more particularly to disinfectants, is important from several standpoints. There is,

for example, a considerable divergence of opinion concerning the efficacy of dead and live vaccines. These differences are based on different results obtained by various authors, which may be due, if not entirely, at least in a large measure, to the variation in the character of the materials used, and the failure or inability to establish whether the vaccine was or was not entirely killed. This problem has been dealt with in previous publications (Kligler, 1930, 1931). Another aspect of the problem is the nature of the disinfectant to be used for the destruction of virus. The American Commission for the Study of Foot and Mouth Disease (1928) calls especial attention to the *rôle* of protein in the lethal effect of various disinfectants. The protein factor may also be of equal if not greater significance in any attempt to differentiate viruses by their relative resistance to heat and disinfectants. It is obvious that in virus materials obtained from different sources the protein variable will be greater than in a particular virus suspension, obtained from the same source and prepared more or less in the same manner. For these reasons the data presented below seem to us of interest and, moreover, the procedure developed should prove of value in throwing light on the nature of viruses.

Experimental.

The adsorption and elution were carried out in the manner previously described. The adsorption was made with 20 per cent. kaolin at ice-box temperature for 24 hours. The phage elution was made with a N/100 solution of ammonia, and the fowl-pox virus with a N/500 and N/1000 $NH_4(OH)$ respectively. The eluates were neutralized with M/15 primary phosphate solutions and stored in the ice-box.

For the tests the original stock suspensions which served as control were diluted to the same extent as the eluates in order to obtain comparable suspensions. In one experiment the original bacteriophage suspension was treated with $NH_4(OH)$ in the same way as the eluate in order to ascertain whether the alkali had an injurious effect on the phage, and similarly serum was added to the eluate in order to gauge its protective action. The solutions of the disinfectants were always such as to require 0·1 c.c. to give the desired dilution when added to the virus or phage suspension.

Experiments with a B. coli *Bacteriophage.*

Experiment 1.—In this experiment were tested the relative effects of various concentrations of alcohol, phenol, mercuric chloride and ether. The tests were made with a variety of phage suspensions prepared in different ways:

(1) Ordinary broth phage diluted with 3 parts NaCl.

(2) The same as (1), except that 0·05 rabbit serum was added to 4 c.c. of the broth phage before diluting.

(3) The eluate of the original broth phage.

(4) The eluate of the broth phage after addition of serum.

The Esbach test of the four fluids was: 1, +; 2, +++; 3, —; 4, trace; the number of + signs indicating the relative concentration of protein.

TABLE I.—*The Effect of Various Disinfectants on Protein-containing and Protein-free Suspensions of a Coli Bacteriophage.*

Disinfectant.	Concentration per cent.	Period of exposure (days). 1.				4.				30.			
		1.	2.	3.	4.	1.	2.	3.	4.	1.	2.	3.	4.
Alcohol	50	24	28	3	3	10	6	0	0·2	8·0	3·5	0	0
	80	0	0·25	0	0	..	..	..	..	..	..	..	..
Phenol	0·5	50	50	16	50	50	32	0·7	2	18	32	0	0
	2·0	50	40	2	4	1·4	2·2	0	0	0	0·4	0	0
	5·0	0	0	0	0	..	..	..	..	..	..	..	..
Sublimate	1·0	0	0	0	0	..	..	..	..	..	..	..	..
	0·1	0	0	0	0	..	..	..	..	..	..	..	..
	0·01	12	12	0	0	1·5	2	0	0	0·2	0·4	..	..
Ether	..	20	24	0·5	2	24	24	0	0	4	2	..	..
NaCl	Control	50	50	20	40	35	28	17	16	24	32	2·1	12

1 = bouillon; 2 = bouillon + serum; 3 = eluate from 1; 4 = eluate from 2. The numbers indicate millions of phage plaques in 1 c.c. The eluate in the 0·5 per cent. phenol still contained active phage on the 11th day.

The dilutions of the various disinfectants used and the results are shown in Table I. The numbers represent millions of phage plaques in a cubic centimetre of suspension as counted on agar plates. Plates were streaked first with a *coli* culture in the usual way, and then 0·1 c.c. of various dilutions of the phage suspension spread over the entire surface of the plate. The plates were incubated at 37° C. for 24 hours and the plaques counted.

The results (see Table I) show clearly the influence of the presence of protein on the effect of disinfectants. In some cases, especially in the more dilute solution of the disinfectants, the differences are really striking. The action of phenol and ether is particularly interesting because of their use in the preparation of virus, more specifically rabies, vaccines.

Experiment 2.—This experiment differs in some respects from the previous one. Plain broth phage and eluate were used as test suspensions and formalin as the disinfectant. Also the phage suspensions were diluted, the broth with broth and the eluate with saline, so that the concentration of phage at the outset of the experiment was approximately the same in both sets of tubes. Otherwise the technique was the same as above.

Here again there were marked differences in the rate of destruction of the phage in the two suspensions. A 1/500 solution of formalin destroyed in an hour 75 per cent. of the phage in the broth suspension and over 95 per cent. in the eluate. A 1/1000 solution of formalin destroyed in one hour 70 per cent. of the phage in broth suspension and 95 per cent. of the phage in the eluate suspension; in two hours the broth suspension still contained 10 per cent. active phage, while the eluate no longer contained active phage. It is apparent, therefore, that the relatively small amount of protein present in the broth had a marked protective effect on the phage against the action of formalin.

Experiments 3 *and* 4.—The next experiments were designed to gauge the injurious effect of the $NH_4(OH)$ in the elution process, and the protective

TABLE II.—*Effect of the Addition of Serum to the Eluate on the Resistance of Phage to Disinfectants.*

Phage suspension.	Disinfectants.*			
	Alcohol 50%.	Phenol 2%.	Sublimate 1/10,000.	NaCl control.†
1. Bouillon phage. . .	2·2	4·6	7·4	16·0
2. Bouillon + serum (0·05 c.c. serum to 4 c.c. broth) .	10·0	16·0	6·0	16·0
3. Eluate from No. 1 . .	0·3	0·60	0·03	14·0
4. ,, No. 2 . .	2·3	2·6	0·1	10·0
5. No. 3 + 0·05 c.c. serum added to 4 c.c. eluate .	16·0	5·0	3·2	12·0
6. No. 4 + 0·05 c.c. serum added to 4 c.c. eluate .	16·0	12·0	5·4	10·0

* The counts were made after 48 hours' contact with the disinfectants. The numbers represent millions of phage plaques in 1 c.c. solution.

† The control consisted of the particular phage suspension to which NaCl solution was added in place of the disinfectant.

effect of serum on eluate. In Experiment 3 the broth phage was treated with NH_4OH for a few minutes and then neutralized with M/15 primary phosphate. In Experiment 4 the ammonified broth was kept for 48 hours before neutralization.

The disinfectants used were: Alcohol 50 per cent., phenol 2 per cent. and sublimate solution 0·01 per cent. The time of contact was 48 hours.

The experiments indicated that ammonia in the concentration used had no deleterious effect on the potency of the phage. On the other hand the addition of serum to the eluate protected the phage against the action of the antiseptics used. The effect of the serum is illustrated by the data shown in Table II.

Experiment 5.—Another way of testing the protective effect of serum was to expose a broth phage diluted with serum solutions to the action of disinfectants. A broth phage was diluted ten times with the following solutions:

1. With broth,
2. With a 25 per cent. serum broth,
3. With a 50 ,, ,,
4. With a 75 ,, ,,

and the various mixtures exposed for the same length of time to the action of 50 per cent. alcohol, 3 per cent. phenol and 1/10,000 sublimate solution. After contact for 48 hours with the disinfectant, plates were made and the phage plaques counted. In case of alcohol no appreciable differences were noted between the serum-free and serum-containing suspensions. The protective effect of the serum was well demonstrated by the action of phenol and sublimate solution respectively. In the broth suspension 65 to 70 per cent., in the first two serum suspensions 50 per cent., and in the last serum suspension only 5 to 25 per cent. of the phage were destroyed. It is apparent that the greater the amount of serum present the less effective is the action of the disinfectants used.

Experiment 6.—In this experiment we tested the effect of oxidizing and reducing agents. The procedure was the same as above, a comparison being made between a broth phage and its eluate.

The results of this experiment are given in Table III. The findings

TABLE III.—*Effect of Hydrogen Peroxide and Sodium Bisulphite on Eluate and Broth Phage Respectively.*

Disinfectant.	Concentration per cent.	Exposure time (hours). 1. Broth.	1. Eluate.	24. Broth.
H_2O_2	0·05	12·0	0	1·0
	0·025	16·0	0·1	8·0
	0·0125	12·0	0	14·0
Sodium Bisulphite	0·05	16·0	0·13	0
	0·025	16·0	0	0·8
	0·0125	18·0	0	0·6
NaCl Control	..	16·0	0	16·0

The numbers represent millions of phage colonies per 1 c.c. solution.

correspond with those obtained with other antiseptics. The phage in the protein-free substrate is strikingly more susceptible to the action of hydrogen peroxide and sodium bisulphite than is that in the broth. After 1 hour the eluate phage is practically completely destroyed, while the broth phage remains unaffected.

Experiment 7.—This experiment deals with the effect of temperature on phage in different substrates. At temperatures of 60° C. or over the differences are not marked. Striking differences are noted at 55° C., this temperature sufficing to destroy 80 to 98 per cent. of phage in the eluates in 45 minutes, while during the same time over 50 per cent. of the phage in the broth or broth plus serum remains intact. The results are summarized in Table IV. It is evident from all of these experiments that in so far as this particular bacteriophage is concerned, protein, even in relatively small amounts, protects it against the action of a variety of disinfectants, and at 55° C. against the action of heat as well.

TABLE IV.—*Effect of Heat on Different Suspensions of Phage.*

Time in minutes.	Temperature. Broth.			Eluate.			Broth + serum.			Eluate from broth + serum.		
	55°.	60°.	70°.	55°.	60°.	70°.	55°.	60°.	70°.	55°.	60°.	70°.
5	..	0·3	0·03	..	0·2	0	..	0·4	0·12	..	0·3	0
10	25·0	0	0	8·0	0·02	0	25·0	0·15	0·1	12·0	0	0
15	..	..	0	..	..	0	..	..	0·02	..	..	0
20	24·0	0	0	1·5	0	0	24·0	0·2	0·05	12·0	0	0
30	16·0	0	0	1·0	0	0	16·0	0·04	0	11·0	0	0
45	10·0	..	..	0·3	..	..	12·0	0	..	3·0	0	..
60	8·0	..	..	0·2	..	..	12·0	0	..	0·5	0	..
75	9·0	..	..	0·3	..	..	8·0	..	..	0·45	..	..
90	9·0	..	..	0·2	..	..	8·0	..	..	0·5	..	..
105	8·0	..	..	0·04	..	..	8·0	..	..	0·5	..	..
120	6·0	..	..	0	..	..	6·0	..	..	0·2	..	..
Control	..	23·0	..	..	14·0	..	..	25·0	..	..	14·0	..

Numbers represent millions of phage colonies in 1 c.c. solution.

EXPERIMENTS WITH FOWL-POX VIRUS.

The experiments with bacteriophage are in some respects more satisfactory than those with virus. The conditions are more constant. The broth medium is a more uniform substrate than the virus suspension, which may have different concentrations of protein according to the manner of preparation. Moreover, the phage lends itself to dilution and counts, and consequently the progress of the destruction can be followed more or less quantitatively. In the case of the virus, however, the differences had to be marked enough to be measured qualitatively, because the scarification method of testing for the presence of virus does not lend itself satisfactorily to titration. Nevertheless, the results, particularly in so far as some of the disinfectants are concerned, demonstrate clearly the protective effect of the protein present.

TABLE V.—*Effect of Disinfectants on Fowl-pox Virus Suspension in Protein-Containing and Protein-free Substrates.*

Disinfectant.	Dilution.	Time of exposure. 5 hours. Original suspension. Size of lesion and incubation days.	5 hours. Eluate. Size of lesion and incubation days.	8 hours. Original suspension. Size of lesion and incubation days.	8 hours. Eluate. Size of lesion and incubation days.	24 hours. Original suspension. Size of lesion and incubation days.	24 hours. Eluate. Size of lesion and incubation days.	9 days. Original suspension. Size of lesion and incubation days.	9 days. Eluate. Size of lesion and incubation days.
Ether	..	++ (4)	−	++ (5)	−	+ (4)	−	..	..
Formalin	1/500	+ (5)	−	−	−	−	−	..	..
	1/1000	++ (4)	−	++ (5)	± (8)	−	−	1 ves. (8)	−
Phenol	0·5%	..	..	..	..	++ (4)	++ (4)	± (6)	−
	1·0%	..	..	..	..	++ (4)	++ (4)	+ 5	−
NaCl	..	++ (4)	++ (4)	++ (5)	+ (5)	++ (4)	++ (4)	Contaminated	+ (7)

Note.—Since different chickens had to be used for the different parts of these experiments, some allowance has to be made for individual variations in resistance. As a rule only young susceptible leghorns were used in these tests. The signs +, ++, +++ indicate the relative size of the lesions. The number in parenthesis represents the incubation period.

The technique was the same as that employed in our previous experiments. Stock saline suspensions of virus were prepared from crusts of fresh lesions by thorough trituration with glass. Parts of this suspension were treated with 20 per cent. kaolin and eluted with N/500 and N/1000 NH_4OH respectively. The eluates were neutralized with a primary phosphate solution as in the case of the phage. The respective virus suspensions were then treated with the disinfectants in the same manner as the phage, and two standard nicrome loops-full of each material rubbed into a scarified area on the comb or wattle of a susceptible leghorn cockerel. The points of inoculation were examined daily for the appearance and size of the lesion, the incubation time and relative size of the lesion serving roughly as an index of the quantity of virus present.

TABLE VI.—*Effect of Disinfectants on Virus Suspension in Protein-containing and Protein-free Substrates. Time of Exposure 30 Minutes.*

Disinfectant.	Dilution.	Original suspension. Size of lesion and incubation days.	Eluate. Size of lesion and incubation days.
Alcohol	50%	—	—
,,	25%	+++ (5)	+ (7)
Sublimate	1/50,000	—	—
,,	1/100·000	+ (8)	—
Hydrogen peroxide	0·03%	—	—
,,	0·001%	+++ (5)	1 vesicle (9)
Sodium bisulphite	5%	—	—
,,	0·5%	+ (5)	—
NaCl Control	..	+++ (5)	++ (6)

TABLE VII.—*Effect of Heat on Protein-containing and Protein-free Virus Suspensions.*

Temperature, °C.	Time of exposure (minutes). 5. Virus suspension. Size of lesion and incubation days.	5. Eluate. Size of lesion and incubation days.	10. Virus suspension. Size of lesion and incubation days.	10. Eluate. Size of lesion and incubation days.
50	+++ (3)	++ (4)	++ (4)	++ (4)
55	++ (4)	++ (4)	+ (5)	+ (5)
60	± (4)	+ (5)	± (5)	± (7)
65	± 1 vesicle (7)	—	—	—
70	—	—	—	—
Control (not heated)	..	..	..	..
Virus suspension	..	..	..	+++ (4)
Eluate	..	..	..	++ (5)

The results of the experiments are summarized in Tables V, VI and VII. Solutions of 50% and 75% alcohol as well as of 1/10,000 and 1/50,000 sublimate destroyed the original suspension as well as the eluate in 5 hours. Of particular interest are the differences in the effect of formalin, ether and phenol. Formalin destroyed the virus rapidly, the eluate suspension being killed in 5 hours and stock virus in 8 hours, whereas in the case of phenol the virus was still active after 9 days.

The protective effect of the protein is strikingly demonstrated in the case of ether ; in the eluate the virus was dead after 5 hours' exposure, while in the stock suspension the virus was still alive after 24 hours. Similar results were obtained with phenol. If other viruses react in the same manner, it is apparent why different authors report different results with formalin-, ether- or phenol-treated vaccines, and why the personal factor plays so important a part in the preparation of vaccines of uniform strength.

It is of interest to note (1) the selective action of various disinfectants on the purified bacteriophage and pox virus respectively, and (2) the similarity of the reaction of the phage and virus to disinfectants in general. It will have been noted that while the virus is more highly susceptible than the phage to all agents, both of them manifest greater resistance to phenol and ether than to other agents. They differ, however, in their sensibility to alcohol and sublimate ; the virus is extremely sensitive and is rapidly destroyed by these chemicals, while the phage is less affected by them than by the other agents.

SUMMARY.

Data are presented showing that the effect of heat, and more particularly that of various disinfectants on a bacteriophage and fowl-pox virus, is weakened by the presence of protein. There is a general similarity in the reaction of the pure fowl-pox virus and bacteriophage to disinfectants, but there are differences in their relative susceptibility to one or another antiseptic. Both pure virus and pure phage are less sensitive to the action of phenol and ether than to any of the other reagents used.

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EXPERIMENTAL OBSERVATIONS WITH LOW-DOSE RADIATION.

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THE question whether radium possesses any growth-promoting powers has attracted the attention of investigators ever since the biological effects of radium were first observed. Numerous somewhat disconnected examples of temporary growth acceleration or increased metabolism under its influence have been recorded. In recent years clinical records have contained definite cases of malignant disease associated with the use of radium, whilst the experimental induction of neoplasia has been successfully achieved in a series of animals by the implantation of radium. In view of this latter evidence radium must be included in the category of proved carcinogenic agents; the mechanism of its action remains debatable, but appears to be governed by one of two broad principles.

It is possible that radium possesses "specific growth-promoting powers." Regaud (1927), in considering the action of radium, draws a parallel from the law of Arndt-Schulz; in the descending scale of dosage one passes from the destructive action of large doses, through an intermediate zone of neutral growth effect, to a zone of low dosage associated with growth promotion. This idea appears to postulate a certain range of dosage, characteristic of each tissue, in which *direct* stimulation of growth occurs. This conception elevates radium to a very special position with regard to growth, a position which the destructive action alone would not warrant, for a variety of non-specific objects can wreck a delicate piece of mechanism, whereas only special tools can increase its efficiency.

The alternative view attributes any growth-promotion to the reaction of living tissue to slight injury produced by radium. With suitably graded doses radium inflicts a prolonged but recoverable injury on a tissue which, in the process of recovery, exhibits the usual biological tendency to over-correction in the form of hyperplasia and neoplasia. This principle of "indirect growth promotion" brings radium into line with the general group of carcinogenic irritants.

To establish the principle of "direct growth promotion," it would appear necessary to produce definite evidence of increase in growth without any signs of concomitant injury; for each tissue concerned the lowest limit of traumatic dosage should be determined and experiments carried out below that limit. Loeb (1922) has collected numerous examples of temporary acceleration of growth, *e. g.* the increased rate of seed germination described by Gager (1908), the shortening of the rest period of winter buds observed by Weber (1922),

the acceleration of the early stages of segmentation of ova recorded by Bohn (1903), but in no case were the lower limits of traumatic dosage determined, in no case was the acceleration maintained, and frequently it was associated with mal-development or death of the tissue. These experimental findings seem to indicate the rapid utilization of the local tissue reserves without any corresponding increase in income to maintain the increased metabolic level. The changes described were never hyperplastic in character ; the tissue simply reached a stage of greater maturity in a shorter time than normally.

The only examples of true increase in growth, where the metabolic level and rate of multiplication are maintained at a higher level, are found in the neoplasia associated with the use of radium. There are three important characteristics of this phenomenon : (*a*) It requires the prolonged use of radium, (*b*) α or β radiation appears to be essential, and (*c*) evidence of concomitant tissue injury, such as chronic dermatitis or fibrosis, is always present. Clinical accounts of radium cancer give histories of ten to twenty years ; Laborde (1931) can find no record of any cases of cancer following pure γ radiation ; chronic inflammatory changes in the exposed tissues are invariably described. In the case of experimental radium cancer, Daels and Biltris (1931) had to embed radium-bearing strips of silk or collodion in rats or mice for six to thirty months ; the earlier tumours seem to be associated with α radiation ; chronic fibrosis round the implanted strip is a constant feature. This picture of radium cancer is much more in keeping with the principle of indirect growth promotion ; the prolonged use of the more irritating types of radiation with definite evidence of local tissue damage appears to be an essential concomitant of the neoplasia produced. Again, we find no example of neoplasia produced with sub-traumatic dosage.

In none of the clinical or experimental findings are any data available which indicate the relationship between the dose of radium employed and the lower limit of traumatic dosage for the tissue concerned ; consequently it is impossible to draw any conclusions as to " direct growth promotion." In order to investigate this point the following experiments have been performed ; the doses of radium employed have been graded so that they lay below the limit of minimal trauma as determined by histological and biological examination. Three different tissues have been examined, and in no case has there been any demonstrable change which could be considered as evidence of increase in growth.

EXPERIMENTS ON MOUSE SKIN.

Considerable information is available as to the normal condition of mouse's skin and the changes associated with hyperplasia and neoplasia in this tissue. It was hoped that a careful study of the histology and biological behaviour of this epithelium when radiated with sub-traumatic doses might throw some light on the possible existence of " direct growth promotion." The skin over the lumbar region was selected for convenience of access ; the radium was applied in the form of platinum needles 0·5 mm. thick, containing 0·6 mgm. Ra. The requisite number of needles were placed side by side on a small applicator of sheet rubber, 1 mm. thick, and the whole strapped firmly in position as a

saddle on the mouse's back. These applicators could be kept in position for as long as ten days, particularly if a little gum was used to anchor the rubber in position.

The first step necessary was the determination of the lower limit of traumatic dosage. It has been shown by Stoël (1928) that a 0·5 mm. platinum needle containing 2 mgm. Ra, when so applied for 48 hours, will produce a slight but definite lesion at the end of about 15 days. Starting with this dose, a series of mice, in groups of six, were given progressively diminishing doses and the effect studied: 1·2 mgm. Ra for 4 days produced definite epilation, in 3 days it produced slight bleaching of the hair, whereas in 2 days no visible effect could be detected. Using this last dose, a series of mice were irradiated and the skin removed for examination at intervals of 1, 2, 3 days . . . up to 3 weeks; in all cases a rectangular strip of skin, 1 by 1·5 cm., was removed from the irradiated area, stretched to its original size on blotting-paper, and then fixed in warm acetic-free Zenker solution. Sections were cut at right-angles to the long axis of the body; three from the anterior, three from the middle and three from the posterior portion of each strip were examined. The sections were stained with iron-hæmatoxylin, hæmatoxylin and eosin or by Feulgen's acid-fuchsin method. The following points were studied in each preparation as affording the most probable indication of any increase in growth activity: the number, position, and type of mitotic figures, the number of layers of epithelial cells, the degree of "packing" of the cells in the basal layer, and the thickness of the keratinized layer.

Mitotic figures.—The average number of mitoses per cm. of section are given in Table I.

TABLE I.—*Number of Mitoses after Irradiation.*

Nature of skin.	Number of sections.	Number of mitoses per cm.
Control	81	4·5
2 days after radiation	45	0·6
3 ,, ,,	45	1·1
4 and 5 ,, ,,	45	0·8
6 and 7 ,, ,,	45	2·6
8 and 9 ,, ,,	90	2·6
10, 11 and 12 ,, ,,	63	4·2
13, 14 and 15 ,, ,,	54	4·1
16, 17 and 18 ,, ,,	81	3·9
19, 20 and 21 ,, ,,	36	3·8

After a temporary diminution in the number of mitotic figures, the count appears to reach a stationary level about the tenth day. The significance of the mitotic count is rather uncertain without any information as to the corresponding times of mitosis; recent work by Canti and Spear (1930) suggests that this is not appreciably affected by low-dose radiation, so that the return of the mitotic count to the normal level would indicate a normal number of cells in the process of division. There is no evidence of any increase in the number of dividing cells during the period studied.

The type and position of the mitoses showed no marked difference between radiated skin and controls; occasionally mitoses were seen superficial to the basal layer of epithelium, but this was not a striking feature, as might be expected if the cells generally had been stimulated to increased activity.

Thickness of epithelium.—The major portion of the epithelium in normal mouse skin is one cell thick, but quite extensive areas of two or three layers are frequently found. An attempt was made to classify the sections according to the average numbers of layers; a comparison of such measurements showed no difference between the radiated and the controls. In like manner a close similarity was found when the thickness of the keratinized layer was compared. The close "packing" of the cells of the basal layer, a very striking feature in the early stages of hyperplasia of this tissue, was rarely found.

Thus the histological findings confirm the observation that the dose employed was "sub-traumatic," but they afford no evidence whatsoever of any increased activity in the epithelium concerned, *i.e.* there is no evidence of any "direct growth promotion."

It was thought possible that, despite the absence of any direct evidence of increased growth activity, the potentiality of the cells for growth might be increased. To test this point, three series of mice were given the standard "sub-traumatic dose," and the radiated area was then treated with carcinogenic tars of varied potency. This method has been used by Roussy, Lerroux and Peyer (1924), but the doses of radium employed were definitely ulcerative. Three different tars were used—an ethereal extract of gas-tar of high carcinogenic power and two low-temperature tars of low potency; the tar was applied at weekly intervals to clipped areas in the lumbar region which had been irradiated.

TABLE II.—*Results of Combined Radiation and Tarring.*

Tar.	Number of mice.	Treatment.	Number of tumours.
Ether ext.	43 controls	Weekly tarring	10 in 20 weeks
Gas tar.	28 radiated	3 radiations in 1st, 5th and 9th week of tarring	1 in 20 weeks.
K.S.G.	41 controls	Weekly tarring	3 in 35 weeks.
Low temp.	35 radiated	1 radiation at the commencement of tarring	No tumours.
Barnsley	31 controls	Weekly tarring	1 in 38 weeks.
Low temp.	20 radiated	1 radiation at 6th week of tarring	No tumours.

Far from exhibiting any tendency to increased tumour formation, the irradiated skin is clearly less susceptible to the carcinogenic action of the tar.

EXPERIMENTS ON RABBITS' LUNG.

This tissue was selected for various reasons. The pleural surface of the lung offers a flat sheet of cells suitable for uniform radiation. The early signs of hyperplastic response in this layer have been studied in detail by Young

(1930), so that data were available as to the type of change which might occur. Pulmonary epithelium possesses a high degree of radio-resistance, and it was hoped that a correspondingly wide range of sub-traumatic dosage would be available.

Needles containing 1·8 mgm. Ra screened with 0·5 mm. platinum were used. These were applied at two distances for different periods to afford a wide range of intensity and time in dosage. For surface application, the needles were placed on pads of sorbo rubber, 0·75 mm. thick, the fur was clipped over the right thoracic wall, and the whole applicator strapped firmly over the lower ribs in the axillary line. For closer application the animals were anæsthetized and the needles inserted in the intercostal spaces, care being taken not to perforate the parietal pleura. The animals were killed at intervals from 2 days to 2 months after radiation and the lungs examined.

For intercostal needling doses ranging from 144 to 600 mgm.-hours were employed on 12 animals without any visible effect on the pulmonary epithelium. For surface application, doses ranged from 144 to 4500 mgm.-hours in 24 animals; no effect was noted until the highest doses were reached. In two animals, which received 4500 mgm.-hours, the lungs showed desquamation of the epithelium, capillary hæmorrhages and perivascular infiltration.

Thus, over a wide range of increasing dosage, no change in the pulmonary epithelium could be detected until the level of traumatic dosage was reached, *i. e.* again there was no evidence of "direct growth promotion."

EXPERIMENTAL EMBRYOMATA IN RABBITS.

It was thought that embryonic tissue, in virtue of its high growth capacity, might provide suitable material for this investigation. Sixteen-day rabbit embryos were minced in sterile saline and half an embryo was injected into the right erector spinæ of twelve rabbits. The site of injection was chosen as it offered an easily accessible firm muscle mass into which the embryo pulp could be introduced under a certain amount of tension: the embryonic grafts appear to take far more satisfactorily in a solid medium than in lax connective tissue. Four of the animals were kept as controls; four animals received a surface application of 7·2 mgm.-hours at 5 mm. distance once a week for three months; the remaining four received 14·4 mgm.-hours under similar conditions. A record of the size of the tumours was kept for one year and the animals were then killed.

In all the animals, radiated and controls, the embryomata enlarged slowly during the first two months, and then remained stationary. Histological examination revealed nodules of cartilage and connective tissue; the cells appeared quite healthy and the tumours had a good blood-supply. The small doses of radium employed appeared to have been quite ineffective, as no difference could be detected between the radiated and the controls.

SUMMARY.

Within the limited range of these experiments, no evidence whatsoever has been found to suggest any increase in growth activity when the doses of

radium are kept below traumatic limits. The tissues selected are all capable of hyperplastic response to other methods of treatment, and their failure to do so under the conditions of these experiments is not without significance. Taken in conjunction with the conditions associated with the production of radium-cancer, these findings do not support the belief in the possession by radium of " direct growth-promoting " properties.

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FILTRATION OF THE VIRUS OF FOOT-AND-MOUTH DISEASE THROUGH A NEW SERIES OF GRADED COLLODION MEMBRANES.*

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Foot-and-mouth disease was the first human or animal disease which was shown to be caused by a filterable virus (Löffler and Frosch, 1898). It is generally recognized that the virus passes through earthenware filters such as the Berkefeld V and Mandler filter candles, the more permeable porcelain filters such as the Chamberland L 1 and L 5 candles and a Seitz asbestos filter disc without any great loss of titre. As regards its high degree of filterability, the virus resembles very closely the bacteriophage and the virus of tobacco mosaic.

The fact that Levaditi, Nicolau and Galloway (1926) showed that the virus of foot-and-mouth disease would pass through alcohol-ether-collodion sacs, permeable to amino-acids, peptones, some protein and bacteriophage, but

* This work was done on behalf of the Foot and Mouth Disease Research Committee who have given their permission for the publication of this paper.

which held back complement, tetanus and diphtheria antitoxins and trypsin, suggested that its order of size is relatively low. Olitsky and Boëz (1927) used Bechhold ultra-filter membranes in an endeavour to arrive at an estimate of the size of the virus. They made a rough estimate that the size of the virus lay between 20μμ and 100μμ by comparison with collargol particles and the relatively large particles of colloidal arsenic trisulphide. More recently Modrow (1929), who also used Bechhold ultra-filter membranes, arrived at the conclusion that the order of size of the particles of the virus of the Vallée and Carée " O " type lay between that of an hæmoglobin particle and that of an egg white or litmus particle, according to Modrow's calculations about 2μμ to 3μμ, and was less than that of the virus of the Vallée and Carré " A " and Waldmann and Trautwein " C " types.

Elford (1931) has recently described a method of preparation of graded collodion membranes of excellent uniformity suitable for general bacteriological use, and of particular service in investigations on filterable viruses. A wide range of permeabilities is available, from a porosity of 3μ down to 10μμ or less ; these membranes may be sterilized by steaming. The present paper describes experiments with such membranes in investigating the filtering properties and order of size of the virus of foot-and-mouth disease. A study has also been made of various fundamental filtration phenomena.

GENERAL TECHNIQUE.

Membrane constants.—The membranes are graded in terms of their average pore diameters (A.P.S.), expressed in microns (μ) or milli-microns (μμ), which are calculated according to Poiseuille's law from data obtained by measuring the rate of flow of water through the membranes under standard conditions (Elford, 1931). The thickness of the membranes used has ranged from 0·13 mm. to 0·25 mm. In general the thickness decreases as the scale of membrane permeability is descended, and it is important that it should be recorded in each experiment. Certain experiments were made to determine the effect on the filtration process of varying the thickness of the membranes ; the data of these experiments are given in a later section. The area of the filtering surface of membrane was 1·05 sq. cm.

Method of mounting the membranes and filtration pressure.—The membranes were supported on a perforated plate, and filtration conducted under positive pressure as in the technique described by Elford (1929). The filtration pressures employed were 30 cm. H_2O, 20 cm. Hg., 76 cm. Hg. and 228 cm. Hg. (3 atmos.).

Quantity of filtrate collected and filtration time.—The amount of stock filtrate used to charge the filter in each experiment, unless otherwise stated, was 6 c.c., and the amount of filtrate collected for titration and other tests, 5 c.c.

The time taken to filter a given quantity of stock filtrate through the membranes, under a given pressure, increased as the scale of permeability was descended. Thus while 5 c.c. of filtrate could be collected in a few minutes under a pressure of 30 cm. H_2O, using a membrane of an A.P.S. of 0·65μ, several hours were necessary with a membrane of an A.P.S. of 40μμ. The

filtration time is also dependent upon such factors as temperature, pressure and membrane thickness. All our experiments were made at a room temperature of 18°–20° C., so that the question of variation in temperature does not come under consideration.

Preparation of stock filtrate.—Guinea-pig vesicle lymph, after being drawn off, was immediately diluted 1 : 10, either in M/25 buffered phosphate saline at pH 7·6 or in Hartley's broth at pH 7·6, this reaction being the optimum for the survival of the virus. This diluted virulent lymph was first subjected to preliminary filtration through a membrane of relatively large A.P.S., *i. e.* 0·5μ to 0·8μ, to remove leucocytes, cell *débris* and any possible bacterial contaminant. A clear, limpid, slightly viscous virus suspension was obtained. The titre of this stock filtrate in the majority of the experiments was 1 : 500,000. In some cases it was between 1 : 100,000 and 1 : 500,000. This preliminary filtration undoubtedly simplified and speeded up the filtration process in subsequent experiments with less permeable membranes.

Titration methods.—The stock emulsion or filtrate to be tested was diluted in series in M/25 buffered phosphate saline at pH 7·6, if the original stock emulsion was prepared in that medium, or in Hartley's broth at pH 7·6 if the original stock emulsion was prepared in broth. Samples of each dilution were immediately inoculated intradermally by several needle-tracks into the pads of the hind feet of each of two guinea-pigs. In certain experiments, where the least permeable of the membranes of the series were being used, more than two guinea-pigs were inoculated with the filtrate to obviate the error of neglecting the important factor of the varying susceptibility of the experimental animal to relatively small amounts of virus. The criterion of the production of the experimental disease in the guinea-pig, with dilutions of emulsions or filtrates, was the appearance of primary and later secondary lesions, *i. e.* a generalized infection.*

Strains of virus employed.—We have tested the filtering properties of five different strains of virus, three of these being of the Vallée and Carré " O " type, G.F., P. 85 and R.A., one of the Valleé and Carré " A " type, R.B., and one of the Waldmann and Trautwein " C " type, R.C.† We have found that all the five strains, which have been adapted to guinea-pigs, titre regularly in healthy members of that species to about 1 : 500,000, provided that their virulence is maintained by passage. Occasionally " takes " may be recorded at dilutions of 1 : 1,000,000, 1 : 2,000,000 or even 1 : 5,000,000, but this is not the rule.

EXPERIMENTAL RESULTS.

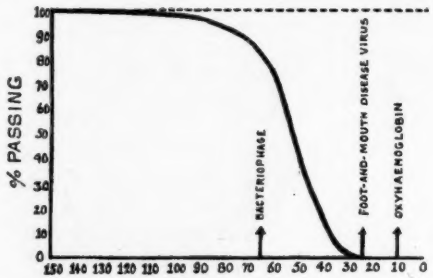
Filtration " end-point " of the virus.

An attempt has been made to arrive at an estimate of the size of the virus. One hundred and forty-three experiments have been made. The technique

* For a description of the clinical picture of the experimental disease in the guinea-pig, v. Waldmann and Pape (1920), and Gins and Krause (1924).

† The basis of the methods by which the type of strains of virus may be determined is cross-immunity. [For designation of virus types v. Vallée and Carré (1922), Waldmann and Trautwein (1926), Vallée (1927–28), Vallée and Carré (1928).]

employed in these experiments has already been briefly described. A stock filtrate was prepared in either broth or phosphate saline at a pH of 7·4 to 7·6. This stock filtrate was subsequently filtered through standard membranes of varying A.P.S. under different pressures. In Table I we give the results of these experiments made with a strain of virus G.F., of the Vallée and Carré " O " type. It will be observed that our results demonstrate clearly that broth greatly facilitates the filtration of the virus through membranes of A.P.S. below 100 μμ. Ward and Tang (1929) also found that hormone broth definitely helped the passage of the viruses of herpetic encephalitis and vaccinia through earthenware and porcelain filter candles. The effect is undoubtedly due to modification of the surface properties of virus and filter owing to adsorption of certain surface active constituents of the broth. The net result is analogous to lubrication, the frictional forces being minimized. We have recorded the amount of virus passing through each graded membrane as a percentage of the virus contained in the stock filtrate. The successive dilutions in a given series


% PASSING
BACTERIOPHAGE
FOOT-AND-MOUTH DISEASE VIRUS
OXYHAEMOGLOBIN
150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
A.P.S. in μμ
CHART I.

were generally from two to fivefold. The estimation of the percentage was made by taking an average of the titres of the filtrates in the different experiments made with membranes of the same A.P.S. In experiments where animals have to be used as a test for the presence or absence of virus in a high dilution of filtrate, it is obvious that the degree of accuracy to which such estimations can be made is only very relative. For this reason in giving the percentage of virus passing through membranes of an A.P.S. <60 μμ which were obviously holding up some virus, we have stated limits which we consider to represent fairly the results obtained.

Chart I represents diagrammatically the results of filtration experiments given in Table I.

An appreciable drop in filtrate titre is first detected with membranes of an A.P.S. of 60μμ, and the amount of virus passing then diminishes rapidly to the 25μμ " end-point." The initial gradual fall in titre in the region of A.P.S. 100μμ to 60μμ might be attributed to virus particle aggregates being held back. However, in view of the factors governing filtration processes (surface forces, mechanical blocking of pores and the distribution of pore size in

TABLE I.

A.P.S.	Filtration pressure.	Percentage of virus passing membrane. Phosphate.	Percentage of virus passing membrane. Broth.	Bacteriophage (coli) control.	Oxy-hæmoglobin control.
0·65 μ (7 experiments)	30 cm. H_2O	100	100	—	—
	20 cm. Hg.	100	100		
	76 ,,	100	100		
0·4 μ (3 experiments)	30 cm. H_2O	100	100	—	—
	20 cm. Hg.	100	100		
	76 ,,	100	100		
0·26 μ (6 experiments)	30 cm. H_2O	100	100	—	—
	20 cm. Hg.	100	100		
	76 ,,	100	100		
0·15 μ (5 experiments)	30 cm. H_2O	100	100	+	—
	20 cm. Hg.	100	100		
	76 ,,	100	100		
60 μμ (29 experiments)	30 cm. H_2O	*Nil*	10	*Nil*	+++
	20 cm. Hg.	2	20–50		
	76 ,,	10	50–100		
45 μμ (13 experiments)	30 cm. H_2O	—	1–10	,,	+++
	20 cm. Hg.	—	1–25		
40 μμ (18 experiments)	30 cm. H_2O	*Nil*	1	,,	+++
	20 cm. Hg.	,,	1–10		
	76 ,,	,,	1–20		
36 μμ (26 experiments)	20 cm. Hg.	—	1–5	,,	+++
	76 ,,	—	5		
30 μμ (12 experiments)	20 cm. Hg.	—	1	,,	+++
	76 ,,	—	1		
25 μμ (24 experiments)	3 atmos.	*Nil*	*Nil*	,,	++

Remarks.

Titre of stock filtrate 1 : 500,000.
pH 7·4–7·6.
Membrane thickness 0·13–0·25 mm.
Volume of filtrate 5 c.c.
Area of the filtering surface of membrane 1·05 sq. cm.
— = No tests made.

individual membranes), the sharpness of the filtration end-point would suggest that the virus particles are of relatively uniform size.

We have stated that the end-point in membrane permeability for the virus of foot-and-mouth disease was found to be 25μμ. Employing membranes of this grade no virus was detected in the filtrate in those experiments in which two test animals were used. We must add, however, that in three experiments, two with G.F. strain and one with P. 85 strain of virus (both of the Vallée " O " type), in which a larger number of test animals were inoculated with the filtrate, a slight trace of virus could be detected, *i. e.* one guinea-pig out of twelve, one guinea-pig out of twelve and one guinea-pig out of four inoculated with filtrate developed a generalized infection (*vide* Table X). These results can be explained by the well-known varying susceptibility of normal healthy guinea-pigs to a minute quantity of virus. Serum proteins, as indicated by the sulpho-salicylic acid test and by boiling filtrates, were found to pass through all the graded membranes employed.

Control Experiments with Bacteriophage and Oxyhæmoglobin.

Now 25μμ, the filtration " end-point " for foot-and-mouth disease virus, is intermediate between the previously established end-points for bacteriophage (coli) and oxyhæmoglobin (Elford, 1931). Bacteriophage having been shown by one of us (I. A. G., unpublished observations) to have no deleterious effect on the virus of foot-and-mouth disease, it was decided to put phage into our broth virus suspension to serve as a control.

The concentration of bacteriophage in the mixture was invariably 10^9 per c.c., and the filtrates were tested both by inoculating young broth cultures of *B. coli* and by the agar plate culture method. In no instance was phage detected in filtrates, using membranes of an A.P.S. less than 65μμ, while the virus continued to pass down to 25μμ. Again using oxyhæmoglobin as a control in broth suspensions of virus, while the virus was retained at 25μμ, the oxyhæmoglobin passed with little apparent loss as indicated by colorimetric comparison. The end-point for oxyhæmoglobin was 10μμ. These results, obtained under comparable conditions as to medium and applied filtration pressure, were consistent over the pH range 6·5–8·5 within which the virus is stable, so that it appears to be established beyond reasonable doubt that the relative sizes of the dispersed elements concerned are bacteriophage (coli) > virus of foot-and-mouth disease > oxyhæmoglobin.

Experiments to Demonstrate the Effect of Medium on the Filtration of the Virus.

That broth facilitates the filtration of virus through the less permeable membranes of the series of graded collodion membranes has already been indicated. Further experiments were planned with these membranes and also with the Seitz asbestos filter discs to obtain a more quantitative measurement of the effect.

Experiment 1.—*Collodion membrane filtration*: 24-hour guinea-pig vesicle lymph was diluted 1 : 5 in physiological saline and centrifugalized for 5 minutes

at 2400 revs. per minute. The supernatant fluid was filtered through a membrane of A.P.S. 0·65μ under a pressure of 76 cm. Hg. The filtrate so obtained was divided into two portions which were diluted 1 : 5 respectively, in broth at pH 7·6, giving stock filtrate (*a*), and in buffered phosphate saline at pH 7·6, giving stock filtrate (*b*). These stock filtrates, (*a*) and (*b*), were then filtered under strictly comparable conditions, using membranes of an A.P.S. of 45μμ under a pressure of 76 cm. Hg. Samples of the filtrates were collected at intervals during the filtration process, and tested for virus content. The results of the experiment are given in Table II.

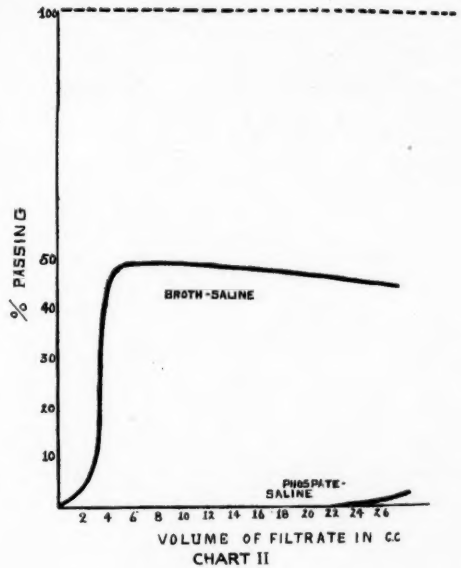


CHART II

In the case of filtrate (*a*) (medium broth) the maximum potency of the filtrate had already been reached when 3 c.c. had been filtered, while in the case of filtrate (*b*) (medium phosphate saline), 25 c.c. of fluid had been collected before any virus was detected. Chart II represents diagrammatically this result.

Experiment 2.—*Seitz filter disc filtration* : 24-hour guinea-pig vesicle lymph was diluted 1 : 5 in physiological saline. This virus suspension was centrifugalized for 5 minutes at 5000 revs. per minute. The supernatant fluid was pipetted off and divided into two portions which were diluted 1 : 5, respectively in broth at pH 7·6, stock filtrate (*a*), and in buffered phosphate saline at pH 7·6, stock filtrate (*b*). Stock filtrates (*a*) and (*b*) were then passed respectively through a Seitz asbestos filter under a positive pressure of 18 cm. Hg. Samples of the filtrates were collected at intervals during the filtration process to test for virus content. The results of the experiment are given in Table III.

TABLE II.

	Successive filtrate samples.						Remarks.
	1 c.c.	2 c.c.	2 c.c.	5 c.c.	5 c.c.	10 c.c.	
(*a*) Broth 4, saline 1	No virus	1 : 1000 ++ 1 : 10,000 +−	1 : 10,000 ++ 1 : 100,000 +−	1 : 10,000	1 : 10,000	1 : 10,000	Membrane A.P.S. 45$\mu\mu$. Pressure 76 cm. Hg. Emulsion made 1 : 5 in saline diluted (*a*) in broth, (*b*) in buffered phosphate saline.
(*b*) Phosphate 4, saline 1	,,	No virus	No virus	No virus	No virus	1 : 100	

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

TABLE III.

	Successive filtrate samples.						Remarks.
	1 c.c.	2 c.c.	2 c.c.	5 c.c.	5 c.c.	5 c.c.	
(*a*) Broth 4, saline 1	1 : 500	1 : 5000 ++ 1 : 50,000 +−	1 : 5000 ++ 1 : 50,000 +−	1 : 50,000 ++	1 : 50,000 ++	1 : 50,000 ++ 1 : 500,000 +−	Seitz asbestos filter disc. Positive pressure 18 cm. Hg. Emulsion made 1 : 5 in saline diluted (*a*) in broth, (*b*) in buffered phosphate saline.
(*b*) Phosphate 4, saline 1	No virus	No virus	1 : 50 ++ 1 : 500 +−	1 : 50 ++ 1 : 500 +−	1 : 5000	1 : 50,000 ++ 1 : 500,000 +−	

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

TABLE IV.

Number of membranes.	Total thickness.	Successive filtrate samples.						Remarks.
		2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	4 c.c.	
(A) 1	0·2 mm.	1 : 100,000	1 : 100,000	1 : 100,000	Not tested	Not tested	Not tested	Membrane A.P.S. 62 $\mu\mu$. Titre of virus 1 : 100,000.
(B) 3	0·6 mm.	*Nil*	1 : 100 ++ 1 : 1000 +−	1 : 100,000	1 : 100,000	1 : 100,000	,,	
(C) 5	1·0 mm.	,,	*Nil*	*Nil*	1 : 10,000	1 : 10,000 ++ 1 : 100,000 +−	1 : 100,000 +−	

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

In the case of filtrate (*a*) (medium broth) some virus could already be detected in the first c.c., and the maximum potency of the filtrate was approached when 10 c.c. had been filtered, while in the case of filtrate (*b*) (medium phosphate saline) no virus was detected until 5 c.c., had been filtered, and the maximum potency of the filtrate was not attained until 20 c.c. had been passed through the filter.

The results of these two experiments demonstrate very clearly the advantage of using broth as a medium for virus filtration work with graded collodion membranes and the Seitz asbestos filter disc—an advantage which has already been proved for the filtration of certain viruses through porcelain and earthenware bacteriological filter candles. Gibbs (1931), using Seitz asbestos filter discs and phosphate saline as the virus diluent in fractional filtration experiments, showed that the first c.c. to be filtered contains very little virus, and that before the maximum potency of the filtrate is attained 20 c.c. to 30 c.c. may have to be collected. Our results recorded in Experiment 2 confirm this observation, in addition to proving the effect of broth on filtration. It is evident, from the data given above, that entirely false interpretations may be given to results of filtration experiments made with virus diluted in saline or phosphate saline, especially if only small amounts of filtrate are collected.

Experiments to Demonstrate the Influence of Membrane Thickness on Filtration.

In dealing with a disperse colloidal system such as a virus suspension, the pore size of the membrane is not the only factor to be considered. Surface adsorption is always a factor exerting an important influence on filtration. It is obvious that if the thickness of the membrane used is increased, the surfaces of adsorption of the capillary channels are also increased.

Experiments were made therefore to study the effect of increase of membrane thickness on filtration.

Experiment 1.—A stock filtrate in broth medium was prepared in the usual way. This titred to between 1 : 100,000 and 1 : 500,000, and was divided into three portions, A, B and C. A was filtered through a single membrane 0·2 mm. in thickness and of A.P.S. 62$\mu\mu$, B through three such membranes, superimposed (total thickness 0·6 mm.), and C through five such membranes, superimposed (total thickness 1 mm.). The pressure employed was 76 cm. Hg. Successive filtrate samples were collected at intervals during the filtration processes. The results of the experiment are given in Table IV.

Filtrate A had attained its maximum potency when 2 c.c. had been filtered, filtrate B when 6 c.c. had been filtered, and filtrate C had not quite attained its maximum potency when 14 c.c. had been filtered. In effect, the surface adsorption factor in filtration is roughly proportional to the membrane thickness. This is further demonstrated by the following experiment:

Experiment 2.—The stock filtrate, in this experiment, titred to about 1 : 500,000. The pressure employed in each filtration process was 76 cm. Hg.

Six c.c. of stock filtrate was first of all filtered through three superimposed membranes of an A.P.S. of 36$\mu\mu$, and of an individual thickness of 0·185 mm. In the 5 c.c. of filtrate collected no virus could be detected. A quantum of

stock filtrate was then filtered successively through three single membranes of similar grade and thickness. The amount of stock filtrate used to charge the first filter was 6 c.c. and the amount of filtrate collected 6 c.c. Of this filtrate A, 0·5 c.c. was kept to test for virus content and the second filter was charged with the remainder. The amount of filtrate B collected after the second filtration was 5·5 c.c.; of this, 0·5 c.c. was put aside to test for virus content and the third filter was charged with 5 c.c. The amount of filtrate C collected was 5 c.c. In Table V we give the results of this experiment.

TABLE V.

Titre of filtrate. 3 superimposed membranes. Total thickness 0·555 mm. 5 c.c.	Titre of filtrates. (A). 1st membrane thickness 0·185 mm. 6 c.c.	(B). 2nd membrane thickness 0·185 mm. 5·5 c.c.	(C). 3rd membrane thickness 0·185 mm. 5 c.c.	Remarks.
No virus	1 : 10,000	1 : 100	No virus	Membranes A.P.S. 36μμ.

Filtrate A titred to 1/10,000, filtrate B to 1/100, and in filtrate C no virus could be detected. The net adsorption in filtering the fluid successively through three single membranes is equivalent to that observed in a straightforward filtration through a membrane of treble thickness.

Effect of Virus Concentration on Filtration.

In our experiments on the filtration " end-point " of the virus, we employed a stock filtrate in which the virus was in high concentration, *i.e.* a 1 : 10 dilution of virus which titred to 1 : 500,000. As it was important to determine the effect on the filtration process of diminishing the concentration of virus in the stock filtrate, a series of experiments was made with membranes of varying permeability. The technique employed in each experiment was essentially the same. The strains of virus used were G.F. of the Vallée and Carré " O " type. The stock broth filtrate was filtered without further dilution (S.B.), diluted ten (S.B./10) or one hundred times (S.B./100) with broth. The filtration pressure was 20 cm. Hg. The amount of filtrate collected in each filtration process was 5 c.c., as in the majority of experiments as yet described. The results of the series of experiments have been grouped together and are recorded in Table VI.

The effect of the concentration of the virus on the filtration process is very marked, especially when the less permeable membranes are in use. In the two experiments, made with membranes of an A.P.S. of 40μμ, only a small amount of virus could be detected in the 5 c.c. of filtrate collected when the stock filtrate was diluted ten times (S.B./10), before filtration, and no virus could be detected in the filtrate when the stock filtrate was diluted one hundred times (S.B./100).

These results show very clearly that it is all-important to determine the

Table VI.

Titre of stock filtrate.	Membrane A.P.S.	Titre of filtrate using (S.B.).	Titre of filtrate using (S.B./10).	Titre of filtrate using (S.B./100).
1 : 500,000	0·26 μ	1 : 500,000	1 : 10,000	1 : 50 ++ 1 : 500 +−
1 : 500,000	62 $\mu\mu$	1 : 250,000 ++ 1 : 500,000 +−	1 : 50,000	1 : 50 ++ 1 : 500 +−
1 : 500,000	48 $\mu\mu$	1 : 10,000 ++ 1 : 100,000 +−	1 : 100 ++ 1 : 1000 +−	1 : 10 ++ 1 : 100 +−
1 : 500,000	40 $\mu\mu$	1 : 50,000 ++ 1 : 100,000 +−	Undiluted ++	No virus
1 : 500,000	40 $\mu\mu$	1 : 10,000 ++ 1 : 50,000 +−	Undiluted ++	,,

Remarks.

S.B. = Stock filtrate.

S.B./10 = Stock filtrate diluted ten times.

S.B./100 = Stock filtrate diluted one hundred times.

Thickness of membrane 0·2 mm. to 0·3 mm.

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

Table VII.

Titre of stock filtrate.	Membrane A.P.S.	Filtration pressure.	Successive filtrate samples. 1 c.c.	2 c.c.	2 c.c.	5 c.c.	5 c.c.
1 : 500,000	40 $\mu\mu$	20 cm. Hg.	No virus	1 : 1000	1 : 50,000	1 : 10,000	1 : 50,000
1 : 500,000	40 $\mu\mu$	76 ,,	,,	1 : 10,000	1 : 10,000	1 : 10,000	1 : 10,000
1 : 500,000	62 $\mu\mu$	20 ,,	,,	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−
1 : 500,000	62 $\mu\mu$	76 ,,	,,	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−	1 : 100,000 ++ 1 : 500,000 +−
			2 c.c.	2 c.c. 2 c.c.	6 c.c.	2 c.c. 6 c.c.	2 c.c.
1 : 100,000*	62 $\mu\mu$	76 ,,	1 : 100	1 : 1000 1 : 10,000 ++ 1 : 10,000 +−	Not tested	1 : 10,000 Not tested	1 : 10,000 ++ 1 : 100,000 +−
			No phage	No phage No phage	,,	No phage ,,	No phage

Remarks.

Thickness of membranes 0·2 mm.–0·25 mm.

*Bacteriophage was mixed with the stock virus filtrate.

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

concentration of virus in any suspension to be subjected to filtration. They also point to the impossibility of drawing conclusions from the results of filtration experiments with two strains of the same virus of different titre, without giving due consideration to this factor. The reason for the effect of virus concentration on filtration being more pronounced when the less permeable membranes are in use should be fairly obvious. As explained in the previous section, specific surface adsorption always plays an important part in the filtration process. The capillary channels become increasingly fine, and the importance of specific surface adsorption is increased in proportion to the increased ratio of surface to volume of capillary as the scale of permeability is descended.

Analysis of Filtration in Terms of Volume of Filtrate Collected.

Attention has already been drawn to the fact that entirely false interpretations may be given to the results of filtration experiments in which strict attention is not paid to the volume of virus filtered (*vide* section on the effect of medium on filtration). The following two series of experiments were made with certain membranes to determine how the virus concentration in the filtrate varied with the volume collected :

Experiment 1.—The stock broth filtrate was prepared in the usual way. The A.P.S. of the membranes used was 62$\mu\mu$ in three experiments and 40$\mu\mu$ in the other two. Successive filtrate samples were collected to test for virus content. The results of the experiment are given in Table VII.

In one of the tests with membranes of an A.P.S. of 62$\mu\mu$ bacteriophage was mixed with the stock virus filtrate, which was then diluted 1 : 5 before filtration. In this test no bacteriophage was detected in any of the successive samples of filtrate, the total amount collected being 22 c.c. Virus was present in the first sample of 2 c.c. collected, and the filtrate had attained its maximum potency for a membrane of this A.P.S. (*i. e.* 62$\mu\mu$) after 4 c.c. had been filtered. In the other four tests, the first sample of 1 c.c. collected contained no virus, but the maximum potency of the filtrate had already been reached when a total of 3 c.c. had been filtered (*cf.* Seitz asbestos filter disc under similar conditions 10 c.c.). The rapidity with which the filtrate attains its maximum titre when using these graded collodion membranes is due to their relative degree of thinness, and in consequence low specific adsorption value. The minimum adsorptive surface which they present gives them a great advantage over earthenware or porcelain filters and Seitz filter discs for virus filtration work.

In the previous section it was shown that when working with a membrane of low permeability, if the concentration of virus in the stock filtrate subjected to filtration is low and only a 5 c.c. sample of filtrate is collected, no virus may be found to have passed through. The following experiment was made to determine whether, if the filtration process was continued, virus would be found to be present in later successive samples of filtrate collected.

Experiment 2.—The stock broth filtrate (S.B.) titred to 1 : 500,000. (S.B.) was filtered without further dilution, diluted ten times (S.B./10) and one hundred times (S.B./100) respectively through membranes of an A.P.S. of 36$\mu\mu$. In

order to magnify the adsorption effect two superimposed membranes of this grade were used in each case. The usual 5 c.c. sample was collected in each case and tested for virus content. The filtration process proceeded, and further successive samples were collected and titrated. The results of the experiment are given in Table VIII. They show quite definitely the importance of taking

TABLE VIII.

Stock filtrate.	Successive filtrate samples.			Remarks.
	5 c.c.	5 c.c.	5 c.c.	
S.B.	1 : 1000	1 : 1 : 1000 ++ 1 : 1 : 10,000 +−	..	Filtration pressure 20 cm. Hg.
S.B./10	No virus	1 : 1000	1 : 1000	Membrane A.P.S. 36 $\mu\mu$.
S.B./100	,,	1 : 10	1 : 10	Membrane thickness 0·37 mm.

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

into consideration the two factors of virus concentration and volume of virus filtered through membranes of standard thickness. Although no virus could be detected in the first 5 c.c. sample of filtrate collected when S.B./10 or S.B./100 were filtered, the virus could be recovered from successive samples of filtrate, *i. e.* the specific adsorbing capacity of the filter had been satisfied only when a larger volume of the lower concentration of the adsorbable constituent had passed through the filter. It should here be noted that we have made a number of experiments employing strains of different types with membranes of an A.P.S. of 25$\mu\mu$, the end-point in membrane permeability for the filtration of the virus. In these experiments we have tested small successive filtrate samples, during the filtration process, for virus content up to a total amount of 8 c.c., 13 c.c., 17 c.c., and even 30 c.c. In none of these experiments was virus found to be present in any of the samples of filtrate.

Effect of Hydrogen Ion Concentration on Filtration.

Olitsky and Boëz (1927) stated that at a pH of 7·5 the virus carries an electro-positive charge, and that in this state the virus is completely adsorbed by the Chamberland L II filter candle, but that at a pH of 8·5 it passes this filter. They stated that at the latter pH the virus carried an electro-negative charge. This observation also led them to employ a medium at a pH of 8·5 in filtration experiments with Bechhold ultra-filter membranes. As regards the electric charge, Sichert-Modrow (1930) and Gibbs (1931) have definitely shown that the virus carries an electro-negative charge at all ranges of pH from 6·5 to 8·6.

The extreme irregularity of results that we have personally obtained with Chamberland filter candles stated to be of the same porosity show the lack of accuracy in standardizing them, and do not lead us to attach importance to any filtration experiments made with them.

We have made, however, a number of experiments with graded collodion membranes to study the effect on filtration of varying the pH of the medium in which the virus is suspended. The technique employed in the first two experiments was essentially the same. Three batches of Hartley's broth and an equal part of M/5 buffered phosphate were made up to different pH. To 12 c.c. of each of these media 2 c.c. of a stock filtrate prepared in the usual way was added, and the pH of each batch was checked electrometrically. It was found that the virus suspensions A, B and C had a pH of 6·43, 7·64 and 8·41 respectively. A, B and C were filtered separately through a membrane of an A.P.S. of 34$\mu\mu$ in the one experiment and of an A.P.S. of 30$\mu\mu$ in the other. The filtration pressure was 20 cm. Hg. Successive small samples of filtrate were collected at intervals during the filtration process and tested for virus content. The duration of each filtration was about 6 hours. The control titration of each batch of stock filtrate was made at the same time as that of the various filtrates.

In the third experiment the medium used was phosphate saline. A stock filtrate was prepared in physiological saline; 2 c.c. of the stock filtrate was added to 12 c.c. of each of three batches of M/5 buffered phosphate saline at different pH's. The pH values were determined electrometrically. The

TABLE IX.

A.P.S. of membranes.	Titre of stock filtrate.	pH.	Titre of successive filtrate samples.		
			2 c.c.	3 c.c.	2·5 c.c.
65 $\mu\mu$ (medium phosphate saline)	1 : 10,000 ++ 1 : 100,000 +−	6·4	No virus	1 : 10,000 ++ 1 : 100,000 +−	1 : 10,000
			2 c.c.	2·5 c.c.	2·5 c.c.
	1 : 100,000	7·6	No virus	1 : 10,000 ++ 1 : 100,000 +−	1 : 100,000
			2 c.c.	3 c.c.	2 c.c.
	1 : 10,000 ++ 1 : 100,000 +−	8·7	No virus	1 : 1000	1 : 1000 ++ 1 : 10,000 +−
			2 c.c.	2 c.c.	4 c.c.
34 $\mu\mu$ (medium broth)	1 : 10,000	6·4	No virus	No virus	1 : 100
			2 c.c.	2 c.c.	3 c.c.
	1 : 10,000 ++ 1 : 100,000 +−	7·6	No virus	1 : 10	1 : 100
			2 c.c.	2 c.c.	3 c.c.
	1 : 10,000	8·4	No virus	1 : 10	1 : 100
			2·5 c.c.	2·5 c.c.	1 c.c.
30 $\mu\mu$ (medium broth)	1 : 100,000	6·4	No virus	1 : 10	1 : 10 ++ 1 : 100 +−
			2·5 c.c.	2·5 c.c.	1 c.c.
	1 : 100,000	7·6	No virus	1 : 1000	1 : 100
			2·5 c.c.	2·5 c.c.	1 c.c.
	1 : 10,000	8·4	No virus	1 : 10 ++ 1 : 100 +−	1 : 100 ++ 1 : 1000 +−

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two developed a generalized infection.

pH of A was 6·40, of B 7·60 and of C 8·75. The technique subsequently employed was as in the two experiments described above. The A.P.S. of the membranes used was 65μμ.

The results of these experiments are given in Table IX.

In these experiments we obtained no evidence that the variation of pH over the range 6·4 to 8·7 has any appreciable effect on the filtration of the virus. Bacteriophage (coli) was added as a control in all the experiments. No bacteriophage was detected in any of the successive samples of filtrate collected.

Filtering Properties of Strains of Virus of Different Types.

In the introduction to this paper we referred to the filtration experiments of Modrow (1929) using Bechhold ultra-filter membranes. Modrow concluded that the size of the virus particles of strain of virus of the Vallée and Carré "O" type was less than that of the virus particles of strains of virus of the Vallée and Carré "A" and Waldmann and Trautwein "C" types. We have tested the filtering properties of five different strains of virus, three of these being of the Vallée and Carré "O" type (G.F., P. 85 and R.A.), one of the Vallée and Carré "A" type (R.B.), and one of the Waldmann and Trautwein "C" type (R.C.).

Strain G.F. was recovered from cattle in Germany by Gins and Fortner in 1922, and has been passaged in guinea-pigs since that date. Dr. Fortner kindly sent the strain to one of us (I.A.G.) in 1926. Strain P. 85 was recovered from cattle in England quite recently, and was kindly sent by Dr. Andrews, of the Ministry of Agriculture's Veterinary Laboratory.

Strains R.A., R.B. and R.C., sent by Dr. Trautwein from the experimental station on the island of Riems, in Germany, were those used by Modrow (1929). We had some difficulty, at first, in getting regular "takes" with the R.C. strain, which we received in the form of epithelium in glycerine from the tongue of infected cattle. After a certain number of passages, however, all five strains titred regularly to about 1 : 500,000.

The conditions under which we made experiments with the five strains of virus were all strictly comparable.

The titre of the stock broth filtrate in each experiment was never less than 100,000. Membranes of four different A.P.S. were used—62μμ, 36μμ, 30μμ and 25μμ. The filtration pressure employed for filtration through the first three membranes was 20 cm. Hg. and through the last membrane 3 atmos. The amount of filtrate collected in every case was 5 c.c. The results are given in Table X. No significant difference in the filterability of the several strains was apparent.

The following experiment was made to confirm these observations. A number of guinea-pigs were inoculated with each of the three strains of virus R.A., R.B. and R.C. All of them developed a generalized infection and were subsequently allowed to recover. One month after the date of infection the guinea-pigs were bled and antisera A, B and C against each of the strains of virus were obtained.

Three batches of formalinized vaccine were also prepared, vaccines A, B and C from lymph recovered from the vesicles of guinea-pigs infected with

TABLE X.

Strain of virus.	Virus type.	Titre of stock filtrate.	Titres of filtrates. A.P.S. = 62μμ. t = 0·2 mm. p = 20 cm. Hg.	A.P.S. = 36μμ. t = 0·185 mm. p = 20 cm. Hg.	A.P.S. = 30μμ. t = 0·13 mm. p = 20 cm. Hg.	A.P.S. = 25μμ. t = 0·15 mm. p = 3 atmos.
G.F.	Vallée and Carré "0"	1 : 500,000	1 : 50,000	1 : 10,000	100 ++ 1000 +−	No virus in 10 experiments * A trace in 2 experiments
P.85	Vallée and Carré "0"	1 : 100,000 ++ 1 : 500,000 +−	1 : 10,000	1 : 10,000	No test made	† A trace of virus in one experiment
Riems A	Vallée and Carré "0"	1 : 100,000 ++ 1 : 500,000 +−	1 : 10,000 ++ 1 : 100,000 +−	1 : 1000 ++ 1 : 10,000 +−	1 : 100 ++ 1 : 1000 +−	No virus
Riems B	Vallée and Carré "A"	1 : 100,000	1 : 10,000	1 : 1000	1 : 100 ++ 1 : 1000 +−	,,
Riems C	Waldmann and Trautwein "C"	1 : 100,000 ++ 1 : 500,000 +−	1 : 10,000	1 : 10,000	1 : 100 ++ 1 : 1000 +−	,,

++ = Both guinea-pigs inoculated developed a generalized infection.
+− = One guinea-pig out of two inoculated developed a generalized infection.

* In these two experiments twelve guinea-pigs were inoculated with the filtrate, and one guinea-pig out of the twelve developed a generalized infection.

† One guinea-pig out of four inoculated with filtrate developed a generalized infection.

TABLE XI.

Dilution of filtrate.	Guinea-pigs inoculated with filtrate. Normal.	Protected by antiserum A.	Protected by antiserum B.	Protected by antiserum C.	Protected by antisera ABC.
1 : 10	(1) ++ (2) ++	++	++	++	+0
1 : 100	(1) ++ (2) 00	00	++	++	+0

Dilution of filtrate.	Protected by vaccine A.	Protected by vaccine B.	Protected by vaccine C.	Protected by vaccine ABC.
1 : 10	++	++	++	+0
1 : 100	++	++	++	+0

Guinea-pigs inoculated with virus to control: virus types, vaccines and antisera. Strain of virus dilution 1 : 500.	Protected by antiserum A.	Protected by antiserum B.	Protected by antiserum C.	Protected by antisera ABC.
Virus R A	+0	++	++	+0
Virus R B	++	+0	++	+0
Virus R C	++	++	+0	+0

Strain of virus dilution 1 : 500.	Protected by vaccine A.	Protected by vaccine B.	Protected by vaccine C.	Protected by vaccine ABC.
Virus R A	+0	++	++	+0
Virus R B	++	+0	++	+0
Virus R C	++	++	+0	+0

++ = Primary and secondary lesions (generalized infection).
+0 = Primary lesions only.
00 = No lesions.

each of the three strains. The lymph was diluted 1 : 10 in M/25 phosphate saline at pH 7·6 and filtered through a Seitz filter. The filtered lymph was then treated with 0·1 per cent. formalin at 26° C. for 48 hours. A combined vaccine ABC was prepared by treating a mixture in equal proportions of the three strains of virus with 0·1 per cent. formalin. Lymph from guinea-pigs infected with each of the three strains was collected separately, diluted 1 : 10 in broth at pH 7·6. Equal amounts of three stock broth filtrates prepared in the usual manner from the three strains of virus R.A., R.B. and R.C. were then mixed. The mixture was filtered through a membrane of A.P.S. of 30$\mu\mu$ and thickness of 0·13 mm., under a pressure of 20 cm. Hg. ; 5 c.c. of filtrate were collected. Dilutions of 1 : 10 or 1 : 100 of this filtrate were inoculated intradermally into the pads of (1) two normal guinea-pigs, (2) two guinea-pigs which had received 1 c.c. of antiserum A, (3) two guinea-pigs which had received 1 c.c. of antiserum B, (4) two guinea-pigs which had received 1 c.c. of antiserum C, and (5) two guinea-pigs which had received 1 c.c. of a mixture of the three antisera A, B and C. Similar intradermal inoculations were made into the hind pads of four groups of two guinea-pigs protected respectively against a generalized infection with 0·75 c.c. of vaccine A, vaccine B, vaccine C and combined vaccine ABC.

The results of the experiments are given on the left of Table XI.

All the guinea-pigs developed a generalized infection, except those protected by a mixture of the three antisera or by a mixed vaccine made from the three strains. Primary lesions appeared at the site of inoculation in the latter two groups of guinea-pigs, but no generalized lesions followed. Two guinea-pigs developed no lesions, one of these being a normal guinea-pig and the other a guinea-pig protected by a dose of antiserum A, each having been inoculated with a 1 : 100 dilution of the filtrate. These results show definitely that all three strains of virus had passed through the membrane. In order to make certain that three virus strains were being used, and to control the protective power of the different antisera and vaccines, a second series of inoculations was made. A 1 : 500 dilution of each of the strains of virus was inoculated intradermally into the hind pads of several groups of two guinea-pigs protected by antiserum A, B, C and a mixture of antisera A, B, C, or by vaccine A, B, C or the combined vaccine ABC. The results of these control tests are given on the right of Table XI.

From the results of these experiments we have obtained no evidence that the size of virus particles of any one type is different from that of any other type.

DISCUSSION.

The virus of foot-and-mouth disease is well suited for the study of the several important phenomena underlying membrane filtration. A quantity of virus of great uniform virulence can be easily obtained in the form of lymph from the characteristic vesicles on the pads of the hind feet of guinea-pigs. These vesicles can be produced regularly by inoculation of virulent material intradermally into that site. This lymph, when collected 24 hours after infection, is a relatively clear, highly virulent fluid and contains a very limited amount

of tissue *débris*. To have such a fluid as starting material is an advantage, which is lacking when one is working with a virus which is only present in high concentration in certain particular tissues, as, for example, a neurotropic virus in nerve-tissue. The presence of a large quantity of tissue material in the virus emulsion to be treated greatly complicates filtration. A further advantage in working with the virus of foot-and-mouth disease lies in the availability for titration work of a small experimental animal showing a comparatively slight variation in susceptibility.

The filtration experiments in this paper have had two main objectives: (1) The study of the filtrability of the foot-and-mouth disease virus, together with an estimation of its probable size: (2) the elucidation of the manner in which the several factors concerned in filtration of this type combine to determine the final result. Experimental evidence obtained under strictly comparable conditions has indicated that the virus is intermediate in size between bacteriophage (coli) on the one hand, and the serum proteins and oxyhæmoglobin on the other. This relationship holds for all strains of the virus employed. It must be strongly emphasized, however, that the filtration conditions must be consistent with regard to such factors as virus concentration, the nature and reaction of the medium, thickness of membrane and filtration pressure in order that a reliable comparison of filterabilities may be made. This has been clearly demonstrated by experimental data cited in this paper. Variation in any one of these factors may have a far-reaching effect upon the course of filtration. Failure to appreciate this must lead to misinterpretation of results, and no doubt accounts for much that is inconsistent in previously published work on filtration.

The mechanism of filtration has been studied in reference to simple disperse systems by one of us (W.J.E.). First of all the specific surface affinities of the membrane must come into equilibrium with the solution which is being filtered, and then filtration proceeds through the "equilibrium membrane." As the result of experiments with metal sols whose average particle size has been estimated by methods of ultra-microscopic count, the relationship has been established empirically that a membrane of A.P.S. $< 100\mu\mu$ is able to effectively retain particles of a size one-half to one-third its own pore diameter. A discussion of the manner in which surface forces may operate in determining the permeability of an ultra-filter membrane is beyond the scope of the present paper. One of us (W.J.E.) intends to discuss the question more fully in a paper on the mechanism of filtration. It can be here stated, however, that the above relationship is not inconsistent with what might reasonably be anticipated on theoretical grounds. On this basis the following size values are calculated:

	Particle size.	Remarks.
Bacteriophage (coli) .	20–30$\mu\mu$.	Retained by all membranes of an A.P.S. $< 65\mu\mu$.
Virus of foot-and-mouth disease . . .	8–12$\mu\mu$.	Retained by all membranes of an A.P.S. $< 25\mu\mu$.
Oxyhæmoglobin . .	3–5$\mu\mu$.	Retained by all membranes of an A.P.S. $< 10\mu\mu$.

SUMMARY.

1. The virus of foot-and-mouth disease in broth medium has passed through all graded collodion membranes of an average pore diameter greater than 25μμ, and is effectively retained below this limit of permeability. The size of the virus has been estimated to be 8–12μμ, and has been shown to be intermediate between that of bacteriophage (coli) and oxyhæmoglobin.

2. No evidence has been obtained that the virus of the Vallée and Carré 'O" type differs in size from the Vallée and Carré "A" or Waldmann and Trautwein "C" types as claimed by Modrow (1929). The viruses of all three strains have been found to filter with equal facility when the conditions mentioned in the next sentence are taken into consideration.

3. The influence of medium, variation in membrane thickness and concentration of virus, and applied filtration pressure upon the course of filtration, is discussed.

4. The variation of pH over the range 6·40–8·75, within which the virus is stable, has had no appreciable effect on the filtration of the virus of foot-and-mouth disease.

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SOME DATA CONCERNING THE INFECTIVITY, SURVIVAL AND POWERS OF DIFFUSION OF THE VIRUS OF "LOUPING-ILL."

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"LOUPING-ILL," a malady of sheep, was definitely shown to be a virus disease by Pool, Brownlee and Wilson (1930). Greig *et al.* (1931) and Alston and Gibson (1931) have reported the successful transmission of the disease to mice. Our attempts in the past to cultivate the virus of poliomyelitis were largely frustrated by the necessity of testing all potentially potent materials on monkeys, and it was hoped that similar work with the virus of louping-ill might afford indications for the successful culture of poliomyelitis virus and of neurotropic viruses in general. The present communication deals solely with preliminary investigations of the time of survival of the virus under such test conditions as might possibly be used in cultivation or in storage of cultures, of the possibilities of diffusion of the organism from infected brain into fluid media, and of the limits of infectivity on progressive dilution of the usual infective filtrate. The mouse has been used exclusively as the experimental animal.

Limit of Infectivity on Dilution of a Berkefeld Filtrate.

Throughout this work, except where otherwise stated, we have used an English-Berkefeld filtrate of a 1 per cent. brain-emulsion in hormone broth. Not less than 30–50 c.c. were passed through the candle under a pressure of about 700 mgm. of mercury. In the following experiments progressive dilutions

TABLE I.

Dilutions of a filtered 1 per cent. emulsion.	Number of animals inoculated.	Result with incubation period in days.
Undiluted	8	Six +5, two +6
10^{-1}	2	+5, +5
10^{-2}	2	+5, +6
10^{-3}	5	+6, +6, +7, +8, +9
10^{-4}	5	+6, +7, +8, +8, 0
10^{-5}	6	+8, remainder 0
10^{-6}	7	All 0

+ = development of louping-ill. 0 = no take.

of such a filtrate were inoculated intracerebrally in doses of about 0·05 c.c.; the inoculations were made through a fine intradermal needle, which readily penetrated the bones of the skull between the base of the ear and the middle line. No skin incision was made, but the skin was carefully cleansed with lysol. The results are seen in Table I.

The results show that infection usually follows the inoculation of 0·05 c.c. of a dilution corresponding to 1 part in 1 million of brain substance, and may occur with a dilution of 1/10,000,000. Greig *et al.* (1931) showed that in the mouse the disease might be transmitted by a 1/1000 emulsion of dry and powdered sheep brain; occasionally the titre was as high as 1/10,000. From the method of preparation, their results are probably not strictly comparable with ours. In respect of the very minute amounts needed to produce the disease, the virus may be compared with the foot-and-mouth virus ('First Progress Report of the Foot and Mouth Disease Research Committee, 1925; Olitsky and Boëz, 1927); it is effective in lower dilutions than the majority of neurotropic viruses, and the very high potency of a Berkefeld filtrate argues, perhaps, for its small dimensions.

With very small quantities of virus there was often an appreciable lengthening of the incubation period, which, with the usual dosage, was almost constantly 4–7 days. This lengthening was even more apparent in subsequent experiments. The longest period experienced was 19 days; Greig *et al.* mention an incubation period of 23 days.

Survival of Virus in Filtrates.

A 1 per cent. filtrate was maintained under the following conditions and tested at intervals, with the results tabulated (Tables II–IV):

(*a*) *In the Cold Room* (+4° *C.*).

TABLE II.

	Aërobic.			Anaërobic.		
	7 days.	14 days.	23 days.	7 days.	14 days.	23 days.
1·00%	+7	+9	0	+6	0	0
0·10%	0	0	—	+10	0	0
0·01%	0	—	—	0	—	—

— = not tested.

(*b*) *At Room Temperature.*

TABLE III.

	Aërobic.				Anaërobic.			
	1 day.	2 days.	4 days.	7 days.	1 day.	2 days.	4 days.	7 days.
1·00%	+7	+7	+7	0	+7	+6	+11	0
0·10%	—	+11	—	0	+7	+8	—	0
0·01%	0	+8	—	0	+8	+10	—	0

(c) *At Incubator Temperature* (37° C.).

TABLE IV.

	Aërobic.				Anaërobic.			
	1 day.	2 days.	4 days.	7 days.	1 day.	2 days.	4 days.	7 days.
1·00%	+18	0	0	0	+11	0	0	0
0·10%	0	0	—	0	0	0	—	0
0·01%	0	0	—	0	0	0	—	0

There is no significant difference in the keeping properties of filtrates kept under aërobic and under anaërobic conditions. Filtrates may retain virulence for as long as 14 days at +4° C., for 4 days at room temperature, and for only 1 day at 37° C.

Survival of Virus in Brain Tissue.

Here sterile pieces of brain were kept in Petri dishes at the temperatures shown (see Table V).

The virulence of brain kept frozen at −10° C. remains unaltered over a period of at least 60 days ; in the same time in the cold room definite loss of virulence has occurred. The virus survives but very few days at room temperature. The results at 37° C. are invalidated on account of drying and shrivelling of the brain ; that the virus does survive for limited periods at 37° C. is shown in further experiments in which the brain was kept in a fluid medium. In broth or Tyrode solution virulence was maintained for 48 hours at 37° C., in 50 per cent. glycerine for 24 hours.

Survival of the Virus in Glycerine.

Mouse brains stored in 50 per cent. glycerine in the cold room (+4° C.) were tested at intervals for virulence. Using direct emulsions for inoculation, virus could be detected after 110 days, using filtrates for a much shorter period. The findings are given in Table VI. Alston and Gibson (1931) found sheep brain potent after 7 weeks in glycerine, Pool *et al.* (1930) after 82 days.

Diffusion of Virus into Liquid Media.

In this experiment infected brains were allowed to stand in a tube containing the fluid medium, the top layers of which, removed without disturbing the brain, were tested for the presence of virus (Table VII).

The results afford evidence of diffusion in all circumstances permitting survival of the virus. Clearest evidence was obtained in the case of specimens kept in the cold room, in which the shorter incubation after a longer time suggested progressive diffusion of the infective agent. The findings at room temperature and at 37° C. must be considered in relation to the period of survival of the virus at these temperatures ; even here definite indications of diffusion were obtained. The difficulty of absolute sterility is responsible for the many gaps in the 37° C. column, while the toxicity of glycerine on intracerebral inoculation account for similar omissions where diffusion into this fluid is considered.

Table V.

	− 10° C.			+ 4° C.			Room temperature.				37° C.	
	25 days.	40 days.	60 days.	25 days.	40 days.	60 days.	3 days.	5 days.	8 days.	14 days.	1 day.	7 days.
2·00%	+6, +8	+5, +7	+6	+5	+5, +7	+8, +11	+7, +8	0, 0, 0	+8, 0, 0	0	0	0, 0
0·20%	+6, +7	+7, +7	+6, +6	+6, +9	+7, 0	0, 0	−	−	−	−	0	0
0·02%	+4, 0	−	+8, 0	+11, +16	−	0, 0	−	−	−	−	0	−

Table VI.

Time.	2 per cent. filtrate.	2 per cent. emulsion.
55 days	+6, +6, +7	+5, +5, +5, +5
90 ,,	0, 0, 0, 0	−
110 ,,	0, 0, 0	+14, +16, 0

Table VII.

Medium.	+ 4° C.		Room temperature.		37° C.			
	10 days.	14 days.	2 days.	5 days.	1 day.	2 days.	4 days.	7 days.
Broth	+7, +7, +8	+5, +5, +5	+8, 0	0, 0	−	−	0, 0, 0	0
Tyrode	+7, +8, +8	+6, +6, +7	−	−	+9, +11, +13	+8, +9 +11, 0, 0	−	−
50% glycerine	−	−	0	+5, 0, 0	−	−	−	0

CONCLUSIONS.

On progressive dilution of a 1 per cent. filtrate, infection may still be obtained by the intracerebral inoculation of a strength of 1/1,000,000, and sometimes with 1/10,000,000.

In the storage of infective filtrates, the presence or absence of oxygen makes little difference to survival of the virus; the periods of survival of the virus at different temperatures have been ascertained.

Figures for the survival of virus in pieces of brain tissue kept in Petri dishes at various temperatures or in glycerine in the cold room have been determined.

Clear evidence of the ability of the virus to diffuse into liquid media has been obtained.

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STUDIES ON RAT THYROIDS.

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I. THE EFFECT OF VITAMIN DEFICIENCY.

ADVANTAGE was taken of the fact that experimental work was being conducted by one of us (J. M.) on the vitamin content—especially "A" and "D"—of certain New Zealand butters, to investigate the changes in the thyroids of the rats used, especially as regards weight, iodine content and histological picture. Later, separate experimental work was done to determine more fully the effect of a low iodine intake with adequate vitamin supply.

Methods.

The technique followed for the "D"-deficiency experiments was that of McCollum (1921 and 1922); rats were fed on his No. 3143 diet (a diet relatively rich in calcium) for one month with the addition of the butter to be tested.

Another group had cod-liver oil added to the 3143 diet, while a group with 3143 diet alone formed the " D "-deficient group. For the " A "-deficiency experiments rats were kept from the time of weaning on a basal diet containing casein, starch, " crisco," " marmite " (for vitamin " B "), irradiated cholesterol (for vitamin " D "), and salts (McCollum's No. 185 mixture).

Iodine estimations.—The method used for the estimation of the iodine content of thyroids, foods, etc., was the modification of that of Th. v. Fellenberg employed by Hercus and Roberts (1927). While a few minutes would suffice for the combustion of the small amount of thyroid tissue taken for analysis, several hours were required for the combustion of large amounts of food, etc., such combustions leaving room for the volatilization of a certain amount of potassium iodide. Hence, though the thyroid results are comparable among themselves, there is not the same comparability between thyroids and such materials as foods.

Owing to the readiness with which a small amount of thyroid, *e. g.* 0·0030 gm., would dry in a room, the weighing of the thyroid lobes was carried out as quickly as possible immediately after they were removed from the animal. Iodine content is stated either as milligrammes per gramme (mgm./gm.) of moist gland, as in the case of thyroids, or in microgrammes (γ), a microgramme being 1/1000 of a milligramme.

Histological examination.—Immediately after killing the rat by chloroform, the thyroid was removed, rapidly weighed, one lobe used for chemical analysis, and the other lobe placed at once in the fluorchrome-bichromate-sublimate (F.B.C.) solution of Williamson and Pearse (1923). Sections were cut serially, and alternate slides stained by phosphotungstic hæmatoxylin and by Weigert's iron-hæmatoxylin with picro-fuchsin. Some glands were fixed in picro-corrosive and some in formol, but the F.B.C. fixation with staining as noted gave the best results for cell details and for comparative purposes.

The histological types are described as " colloid," " active " or " mixed." The term " colloid " needs no explanation; it might perhaps be labelled " passive " or " storage " by contrast with the " active," but it seems unnecessary to introduce still another term. " Active " is used instead of and with the same histological significance as the terms " hyperplastic " of Marine, or " secretory " of Williamson and Pearse. The term " mixed " merely indicates that some parts of the gland are of colloid, and some of active type.

General.—The rats were kept in clean cages, in a room at an approximately constant temperature (15° to 22° C.). Where excreta had to be examined they were collected in a pan below the wire mesh floor of the cage, only a glass jar being supplied as shelter instead of the usual wood shaving bedding. Food was supplied in non-collapsible containers and all intake carefully weighed. Water was given in the usual manner by means of an inverted glass flask and tube.

Results.

Controls.—These are shown in Table I (lower part), diet 3143, with vitamin " D " from cod-liver oil or from ergosterol; also two animals on a meat-meal diet. Eleven animals occurred in this group, but two were not examined chemically.

TABLE I.—*Control Series. No Vitamin Deficiency.*

Serial No.	Age (days).	Sex.	Weight (gm.).	Diet and additions.	Remarks.	Thyroid. Total weight. (gm.).	Iodine content (mgm./gm.).	Total iodine (γ).	Histological type.
7	99	M.	88	3143 + 0·6 gm. butter daily	Normal, healthy	0·0073	1·27	9·3	Colloid.
8	99	F.	77	,, ,,	,, ,,	0·0057	1·37	7·8	,,
26	71	M.	80	,, ,,	,, ,,	0·0109	0·45	4·9	,,
27	71	F.	78	,, ,,	,, ,,	..	..	..	,,
28	71	M.	80	3143 + 0·2 gm. butter (A) daily	,, ,,	0·0080	0·42	3·4	,,
29	71	F.	66	,, ,,	,, ,,	0·0087	0·61	3·5	,,
35	66	M.	73	3143 + 0·6 gm. butter (C) daily	,, ,,	0·0033 (slight loss fron drying)	1·71	5·6	,,
36	66	F.	67	,, ,, ,,	,, ,,	0·0052	1·03	5·4	,,
47	66	M.	71	3143 + 0·6 gm. butter (E) daily	,, ,,	0·0056	0·62	3·5	,,
48	66	F.	65	,, ,, ,,	,, ,,	0·0043	0·83	3·6	,,
49	67	M.	73	3143 + 0·2 gm. butter (F) daily	,, ,,	0·0070	0·36	2·5	,,
50	67	F.	63	,, ,, ,,	,, ,,	—	—	—	,,
51	67	F.	67	3143 + 0·2 gm. butter (E) daily	,, ,,	0·0058	0·61	3·5	
Average (of 11)			77			0·0065	0·84	4·8	
5	114	F.	74	3143 + cod-liver oil	Normal, healthy	0·0076	0·65	4·9	Mainly colloid; some "active" areas.
14	102	M.	90	3143 + cod-liver oil; later ergosterol	,, ,,	0·0048	1·31	6·3	Colloid.
15	102	F.	53	Ditto	,, small	0·0028	1·72	4·8	,,
24	71	M.	73	3143+cod-liver oil (4 drops daily)	,, healthy	0·0068	0·92	6·3	Mainly colloid; some "active" areas.
25	71	F.	59	,, ,, ,,	,, ,,	..	..	..	Ditto.
37	66	M.	74	,, ,, ,,	,, ,,	0·0100	0·80	8·0	,,
38	66	F.	56	,, ,, ,,	,, ,,	0·0052	0·57	3·0	,,
45	66	M.	66	,, ,, ,,	,, ,,	0·0055	0·69	3·8	Colloid.
46	66	F.	60	,, ,, ,,	,, ,,	0·0045	0·71	3·2	,,
56	112	M.	168	Meat-meal diet	Normal mixed litters	0·0104	0·78	8·1	Colloid, and some "catarrhal changes present.
57	112	F.	112	,, ,,	large healthy rats	0·0094	..	..	
Average (of 9)			79·3			0·0064	0·90	5·4	

The growth charts of these animals showed a steady rise in weight with age. The weights of the thyroids varied; those glands with active tissue tended to be heavier than those of pure colloid type, relative to their respective body-weights. No. 37 was relatively large gland. In the series shown in the upper part of Table I the source of vitamin " D " was butter in varying but adequate amount. No. 26 was above the average size, but of colloid type, low in iodine content.

Vitamin " D " deficiency.—Table II gives the details of twelve rats on the 3143 diet, *i. e.* lacking vitamin " D." All showed rickety changes anatomically and chemically. In these there was no enlargement of the thyroid, and the histological types were within the range of normal variation, the colloid type predominating, though some showed active areas. The iodine content of the thyroids varied considerably; as usual the lower the iodine content, the more extensive the active tissue in the gland—as pointed out by Marine (1909) and others.

In minor grades of vitamin " D " deficiency, Table III, where to the 3143 diet small amounts of butter were added, there was no thyroid enlargement. The histological type was variable, colloid again predominating with the higher iodine content, active with the lower iodine content.

Vitamin " A " Deficiency.—(Table IV). From three series of experiments, eight rats were selected and the results tabulated. These rats were put on the "A"-deficient diet already mentioned. All showed typical xerophthalmia, and three had purulent lymph-glands in the neck. In three of these (Nos. 18, 54, 55) the thyroid was enlarged and the histological type of gland was mainly colloid.

Because a thyroid is diffusely active it does not follow that it is necessarily pathological, viz. a goitre, unless it is enlarged well beyond the normal average range for body-weight, or is producing effects by reason of its excessive—or diminished—activity.

An attempt was made to chart the iodine contents in relation to thyroid weights, but unless body-weights are also given, such a chart is difficult to follow, and so has not been given, but it was found—as can be seen from the tables—that there was a similar range of variation in the controls and in the groups with "A" and " D " deficiency.

When the diets of these different groups were examined as regards iodine content (Table V), it was found that in the control series the daily intake of iodine per rat was, approximately, 0·29 to 0·90 microgrammes—an amount which later experiments indicated was adequate for the rat. In the vitamin, " D " deficiency series the iodine intake was slightly lower, 0·148 to 0·756 microgrammes, and in the vitamin-" A " deficiency series 0·35 to 0·5 microgrammes—also figures apparently adequate as regards iodine requirements. All these, therefore, had an adequate—though in some cases low—iodine intake, but a variable vitamin intake resulting in all grades of body change from normal to definite vitamin deficiency effects.

As regards histological changes, the thyroids showed considerable variation in the control and deficiency groups, ranging from colloid to active, with all intermediate grades. Tables I, II, III, IV show all types of gland in each group, except in the 3143 series, with adequate amounts of butter—these glands were all of colloid type.

TABLE II.—*Vitamin-"D" Deficiency.*

Serial No.	Age (days).	Sex.	Weight. (gm.).	Diet.	Remarks.	Thyroid. Total weight. (gm.).	Iodine content (mgm./gm.).	Total iodine (γ).	Histological type.
6	114	F.	82	3143, alone	Slight rickets	0·0076	0·21	1·6	Mainly "active."
9	99	F.	72	,, ,,	Definite rickets	0·0063	0·86	5·4	,, colloid.
16	102	F.	70	,, ,,	,, ,,	0·0052	1·04	5·4	Colloid.
17	102	F.	64	,, ,,	,, ,,	0·0058	0·80	4·6	,,
30	72	F.	63	,, ,,	,, ,,	0·0065	0·34	2·2	Mixed colloid and "active."
31	72	F.	67	,, ,,	,, ,,	0·0078	0·33	2·6	Ditto.
32	72	F.	68	,, ,,	,, ,,	0·0066	0·47	3·1	,,
43	68	M.	77	,, ,,	Marked ,,	0·0058	0·75	4·4	Colloid.
44	68	F.	65	,, ,,	,, ,,	0·0068	0·48	3·3	,,
52	67	F.	61	,, ,,	Definite ,,	0·0046	0·94	4·3	Mainly colloid.
53	67	F.	60	,, ,,	,, ,,	..	..	..	,, ,,
59	..	M.	68 (less hind limbs)	,, ,,	,, ,,	0·0099	0·31	3·1 (rest of carcase* = 3·6)	Mixed, largely "active."
58	..	M.	61 Ditto.	3143 + 0·6 gm. tallow	,, ,,	0·0071	0·40	2·8 (rest of carcase† = 6·0)	Mixed, largely colloid.
Average (of 12)			68·16			0·0066	0·57	3·5	

(*) Total iodine in whole carcase of parallel experiment, 6·67γ.
(†) ,, ,, ,, ,, ,, 7·50γ.

TABLE III.—*Minor Degrees of Vitamin-"D" Deficiency. 3143 Diet + Varying Amounts of Butter.*

TABLE III.—*Minor Degrees of Vitamin-"D" Deficiency.* 3143 *Diet + Varying Amounts of Butter.*

Serial No.	Age (days).	Sex.	Weight (gm.).	Diet and additions.	Remarks.	Thyroid. Total weight (gm.).	Iodine content (mgm./gm.).	Total iodine (γ).	Histological type.
3	110	M.	113	3143 + 0·1 gm. butter daily	Definite rickets	0·0087	0·30	2·6	Diffuse "active"; some degeneration.
4	110	F.	81	3143 + 0·1 ,, ,,	,, ,, and pregnant	0·0055	0·66	3·6	Diffuse "active"; slight fibrosis.
33	72	M.	75	3143 + 0·2 gm. butter (C) daily	Definite rickets	0·0061	0·63	3·8	Mixed colloid and "active."
34	72	F.	76	3143 + 0·2 ,, ,,	,, ,,	0·0062	0·57	3·5	Ditto.
39	67	M.	82	3143 + 0·2 gm. butter (A) daily	,, ,,	0·0064	0·61	3·9	,,
40	67	F.	80	3143 + 0·2 ,, ,,	,, ,,	0·0065	0·69	4·5	,,
1	157	F.	111	3143 + 0·2 gm. butter daily	For 2 months slight rickets	0·0068	1·09	7·4	Colloid.
2	157	F.	105	3143 + 0·2 ,, ,,	Ditto	0·0077	0·79	6·1	,,
10	101	M.	91	3143 + 0·1 ,, ,,	For 40 days slight rickets	0·0094	1·15	10·8	,,
11	101	F.	79	3143 + 0·1 ,, ,,	Ditto	0·0072	0·92	6·6	Colloid with small "active" areas.
12	101	M.	81	3143 + 0·2 ,, ,,	Very slight rickets	0·0077	1·39	10·7	Ditto.
13	101	F.	85	3143 + 0·2 ,, ,,	,, ,,	0·0088	0·95	8·4	,,
41	67	M.	82	3143 + 0·2 gm. butter (C) daily	,, ,,	0·0069	0·71	4·9	Mainly "active."
42	67	F.	77	3143 + 0·2 ,, ,,	,, ,,	0·0068	0·57	3·9	,, ,,
Average (of 14)			87			0·0072	0·79	5·7	

TABLE IV.—*Vitamin-"A" Deficiency.*

Serial No.	Age (days).	Sex.	Weight (gm.).	Food and additions.	Remarks.	Thyroid. Total weight (gm.).	Iodine content (mgm./gm.).	Total iodine (γ).	Histological type.
18	152	M.	109	Basal diet + D, less A	Xerophthalmia	0·0113	0·32	3·6	Mixed colloid and "active" in varying amounts.
19	162	M.	77	,, ,,	,,	0·0084	0·57	4·8	
20	152	M.	72	,, ,,	,,	0·0078	0·44	3·4	
21	154	F.	95	,, ,,	,,	0·0069	0·68	4·7	All colloid. In No. 23 epithelium is flat.
22	154	F.	85	,, ,,	Xerophthalmia and purulent glands in neck	0·0070	0·61	4·3	
23	154	F.	85	,, ,,	Ditto and pregnant	0·0060	0·92	5·5	
54	144	M.	123	,, ,,	Xerophthalmia; weakness of legs; abscesses in neck	0·0184	0·26	4·8	Colloid, with occasional "active" vesicle. Much of epithelium is flat.
55	144	F.	103	,, ,,	Xerophthalmia; weakness of legs; no abscesses	0·0120	0·50	6·0	
Average (of 8)			93·6			0·0097	0·54	4·6	

Discussion.

The results showed that definite vitamin deficiency effects had been produced by appropriate diets, and yet in the same time but little thyroid change that could be considered outside the range of normal variation had occurred. In the control group two glands were larger than the average (Nos. 26 and 37, Table I). In 3 (Nos. 18, 54, 55, Table IV) of the vitamin-"A" deficiency group, the glands were definitely large for their respective body-weights. The variation in histological appearances was the same in the controls and in those suffering from vitamin deficiency. The variations in the groups were not seasonal, as they occurred with equal frequency in animals in winter and in summer, nor were they specially related to sex. They appear to be such variations as may occur in any thyroid, and should hardly be regarded as goiterous—except in the few cases mentioned. We did not find the very large thyroids recorded by McCarrison (1930) in his animals on vitamin-deficient diets.

The iodine content of the diets was adequate, though in some samples of diet it was relatively low. The average iodine content of the thyroids (in mgm./gm.), and their total iodine (in microgrammes), varied very little between different groups. In the series with vitamin-"A" deficiency it was lowest in mgm./gm., though the total iodine in the glands is practically the same as in the other series.

There were many individual variations in each group, which may perhaps be explained by such animals getting extra iodine from an unequal distribution of iodine in the food. That this occurred was indicated by the fact that the constituents of a diet varied in iodine content, and it was impossible so to mix such a diet as to ensure an absolutely even distribution of its iodine content in all samples. In the series with vitamin " A " deficiency the iodine content of the glands was low, with one exception, and yet the histological picture was mainly colloid, even where infection had occurred. In this series body-growth was retarded, as shown by the ages and comparative weights (Table IV) ; this may be a factor in explaining the relatively inert appearances in these thyriods.

McCarrison (1927) showed that a diet rich in white flour and poor in vitamins may cause goitre of a " secretory " or " hyperplastic " type in rats, even though a considerable amount of iodine was added in the salt mixture used. Among his rats there was a very high mortality from such causes as " asthma," " pneumonia " and " beri-beri," while the incidence of goitre in the various groups was very irregular. That the diet was inadequate was shown by the failure of growth and, in many cases, loss of weight of the rats.

We feel that our results do not justify us in saying that we have produced any marked or uniform deviation from the normal variation in rat thyroids as the result of deficiency of vitamins "A" or " D "—with the few exceptions noted. It may be objected that the duration of the experiments was too short for definite goitres to develop. That may be so, but the duration was long enough for very definite deficiency effects to be seen in the bodies of the animals.

Summary and Conclusions.

1. The variations in the thyroids of rats fed on diets adequate in vitamins were examined as regards weight, iodine content and histological type.

2. The variations in the thyroids of rats were examined in animals suffering from deficiency of vitamins "A" and "D," and were found to be within the same limits as the controls, with a few exceptions.

TABLE V.—*Iodine Content of Foods in Microgrammes* (γ).

Casein	150	per kilo.
Purified rice starch	10	,,
Crisco	8	,,
Meat-meal	157·5	,,
Yeast (dried)	40	,,
Bread (white) (Oct.)	6·9	,,
,, ,, (Jan.)	4·8	,,
Milk, fresh (Oct.)	6·0	per litre.
,, ,, (Jan.)	4·8	,,
Cod-liver oil	5000	,,
Marmite (Hercus & Roberts)	15	per kilo.
Spinach (dry)	254·5	,,
Butter A	6	,,
,, B	6	,,
,, C	6	,,
,, D	5·5	,,
,, E	6	,,
,, F	6	,,
Rat diet, 3143 (June) average	75·6	,,
,, ,, ,, (Jan.) ,,	14·8	,,
"A" deficiency, basal diet	35·0 to 50·0	,,
"B" diet	4·8 to 6·5	,,
Approximate average intake of iodine per rat per day :		
Diet 3143	0·148 to 0·756.	
"A" deficiency diet	0·35 ,, 0·50.	
Diet 3143 + cod-liver oil	0·29 ,, 0·90 approx.	
Diet "B"	0·048 ,, 0·065.	
,, with iodine added	10 ,, 14.	

II. THE EFFECT OF A DIET ADEQUATE IN VITAMIN, BUT LOW IN IODINE.

We first endeavoured to produce such a diet by selecting the necessary purified elements with a low iodine content. After trying several different diets of known iodine content, we found that the iodine content of the thyroids and of the carcases of the rats was still considerable. The thyroids also were of colloid type, and showed little appreciable variation between those on the diet alone and those on the diet plus added iodine. Another series was then arranged—Series B. We found the iodine content of ordinary white bread and of fresh whole milk was very low (see Table V), and so we adopted the simple diet of equal parts white bread and fresh milk (special diet B). Iodine was added to a control series in the form of 0·0183 gm. KI to the litre of water;

5 c.c. of this solution was added per day to the drinking-water, giving 70 microgrammes of iodine to the cage per day. On this diet young rats throve well and increased steadily in weight; they remained active and healthy.

The combined litters from two does that had been used in a previous experiment—diet without iodine added—to the number of seventeen were used. At first all were undersized and thin, but on the bread and milk they throve.

At first 8 were put in one group and 9 in another; both groups were kept on the bread and milk diet until they were 54 days old, and of an average weight of 48 and 44 gm. respectively. Then to one group (Table VI, Nos. 18, 20, 22, 24, 26, 28) iodine was added to the water as described. Before the iodine was added one rat was killed (No. 13) in the larger group, to equalize the numbers. One was accidentally killed in one group, and again its opposite number was sacrificed to equalize (No. 16). Great care was taken to keep these animals free from all possible contamination by extraneous iodine. They were kept in a separate room apart from other animals, foodstuffs or reagents of any sort. The temperature was kept constant. Table VI gives details of these animals.

In the younger animals the thyroids were small and of colloid type, but as they grew the thyroids gradually changed in those without iodine added, and became of the active type. Those with iodine added remained colloid. The addition of iodine was stopped about the middle of the experiment, and the glands, from being colloid, gradually assumed the active type, till in the last animals both the bread and milk group alone and the group to which iodine had been added previously were similar. The thyroid weights of the animals on bread and milk alone were higher than those of the group to which iodine was added, except towards the end of the experiment, where the thyroid weights of both groups approximated. The same occurred with the iodine content—high in the smaller colloid glands, low in the larger, active glands, approaching the same level in both groups towards the end. Slight variations in weight occurred in animals of the same age, due in part to sex, but sex seemed to have little effect in the thyroid response to low or high iodine. Pregnancy affected the weight in No. 26 (Table VI), which was pregnant when killed. We noticed that those rats which had iodine added not only grew and developed normally, but reproduced, whereas those without iodine did not reproduce, though they grew equally well.

Discussion.

The results of the last series, rats on bread and milk, are similar to those recorded by McClendon (1927). He and others found relatively high thyroid weight to body-weight in animals on a low iodine intake, and the reverse in those which had iodine added.

In this series (Series B) the animals grew normally throughout the course of the experiment, in contrast to the previous series with vitamin deficiencies. It is true the duration of this experiment was longer, but even for the same ages and weights of animals the changes produced by the iodine-poor or iodine-rich diets were more striking and uniform than any produced by deficient vitamin diets.

TABLE VI.—*Special Bread and Milk, with and without Iodine Added.*

TABLE VI.—*Special Bread and Milk, with and without Iodine Added.*

Serial No.	Age (days).	Sex.	Weight (gm.).	Diet.	Remarks.	Thyroid. n.e.	Thyroid. Total weight (gm.).	Iodine content (mgm./gm.).	Total iodine (γ).	Histological type.	Iodine in carcase (γ).
10	47	F.	37	B. and M. only (=low I.)	Putting on weight; small but healthy	Very small, pink	..	..	..	Colloid	3·2
11	47	F.	37	,, ,, ,,	Ditto	..	..	..	..	..	4·95 (= + thyroid).
13	54	F.	57	,, ,, ,,	Active, good condition, growing	Small, pink	..	..	..	Colloid	..
16	74	M.	92	,, ,, ,,	Ditto	Relatively large, pale clear pink	0·0089	0·18	1·6	,,	3·15
17	91	F.	88	,, ,, ,,	,,	Ditto	0·0141	0·17	2·4	"Active"	< 0·4
18	94	F.	96	,, + added I. for 37 days	Healthy, active, fat	Small, clear pale pink	0·0066	1·50	9·9	Colloid	8·06
19	105	M.	143	,, only	,, ,, ,,	Large, pink, soft	0·0172	0·082	1·4	"Active"	3·77
20	105	M.	144	,, + added I. for 48 days	,, ,, ,,	Small, clear pink	0·0072	1·40	10·1	Colloid	15·83
21	118	F.	102	,, only	Good condition; not pregnant	Large, pink translucent	0·0108	0·17	1·8	Mainly "active" some colloid areas	1·33
22	118	F.	119·5	,, + added I. (until 10 days ago)	Ditto	Small, pale greyish pink	0·0069	1·05	7·2	Colloid; a few "active islets"	14·66
23	145	F.	123	,, only	,,	Large, red, right lobe > left	0·0139	0·083	3·0	"Active"	..
24	145	F.	135	,, + added I. (until 37 days ago)	Good condition, recently pregnant	Slightly large, pale pink	0·097	0·925	4·8	Largely colloid with "active" areas	..
25	174	F.	127	,, only	Good condition; not pregnant	Both lobes large, pink	0·0116	0·15	6·5	"Active"	..
26	174	F.	166	,, + added I. (until 66 days ago)	Good condition; pregnant	,, ,,	0·0095	0·64	16·6	"Active" with scattered colloid vesicles	..
27	202	M.	235	,, only	Good condition	,, ,,	0·0162	0·16	6·2	"Active"	..
28	202	M.	218	,, + added I. (until 94 days ago)	,, ,,	,, ,,	0·0125	0·24	9·3	,,	..
Diet only: (average of 7)			130				0·0132	0·14	3·4		
Diet + I.: (average of 6)			146				0·0087	0·96	9·6		

It seems clear that the iodine requirement is low in the rat, very small amounts sufficing for normal thyroid growth and size; somewhere about 5 or 6 microgrammes of iodine per kilo of food seems to be just under the amount necessary to give a small compact gland, when the diet is otherwise adequate. Hellwig (1931) failed to produce any active type of gland in diets "low in iodine," but he does not state what the actual iodine contents were of the diets used.

As McCarrison (1927) points out, figures for rats in one country do not necessarily apply to those of a different country, so that our figures must be taken to apply only to the rats—which were all albinos—acclimatized to laboratory conditions in New Zealand.

TABLE VII.—*Ratios of Total Iodine (mgm./gm.) to Weight of Thyroid and of Weight of Thyroid* × 10,000 *to Body-weight* × 1.

Diet.	Total iodine / weight of thyroid (mgm./gm.).	Thyroid weight × 10,000 / body-weight.
Normal 3143 + cod-liver oil	0·90	1 : 1·234
Normal + butter	0·84	1 : 1·185
Minor "D" deficiency	0·79	1 : 1·200
Major "D" deficiency	0·57	1 : 1·030
"A" deficiency	0·54	1 : 0·960
Series B (bread and milk only)	0·14	1 : 0·985
„ („ „ + iodine)	0·96	1 : 1·678

In order to compare the ratios of iodine content to weight of thyroid and weight of thyroid to body-weight in the different tables, Table VII is given. This shows that the group with deficiency of vitamin "A" and that on the special diet, Series B (bread and milk alone), had a similar ratio of thyroid weight to body-weight, the thyroid being relatively large in both cases, but the iodine content of the group with the "A" deficiency was higher than the Series B (diet alone). The thyroids of the vitamin "A" deficiency group were mainly of colloid type, while the Series B (diet alone) were of the active type. This suggests that there is a reduced need for iodine (possibly related to retarded growth) or some defect in utilizing the iodine where a vitamin deficiency is present, especially a deficiency of vitamin "A." Where there is a definite deficiency of iodine as in Series B (diet alone), the thyroid reacts by changing to the active type in order to utilize at once and put into circulation the small amounts of iodine available. Where iodine is added, as in Series B (diet plus iodine), the glands are of the storing colloid type and small, as little activity is required to keep a head of iodine-rich content available under these circumstances.

(It would have been desirable to have had further series on the same lines as Series B, but owing to the departure of one of us, further work on these lines was held up.)

Summary and Conclusions.

1. Attempts were made to produce a diet adequate for normal growth in the rat, but sufficiently low in iodine to produce thyroid change. This was best achieved by a diet of white bread and fresh milk.

2. After a period rats on this diet developed an active type of gland, low in iodine content, and large relative to the body-weight.

3. The addition of small amounts of iodine to such a diet kept the thyroids small, colloid, and with a rich iodine content.

4. As iodine was again omitted from the diet the thyroids tended to enlarge, and assume the active type of gland similar to those that had no iodine from the beginning.

Acknowledgments.—We gratefully acknowledge our indebtedness to the Board of Industrial and Scientific Research of New Zealand for grants towards the first part of this work, and to the Health Department of New Zealand for substantial assistance in salary and in grants to cover expenses of the iodine investigations; without such help the work could not have been undertaken.

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TAR CANCER IN MICE. III.—AN APPARATUS FOR THE PREPARATION UNDER STANDARD CONDITIONS OF A HIGHLY POTENT CARCINOGENIC AGENT OF LOW TOXICITY.

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MOST workers in the field of cancer research are familiar with the wide variation met with in the carcinogenic potencies of different coal tars and shale oils. Such variations are not unexpected in view of the different types of coal from which the tars are prepared, and the varying temperatures to which they have been subjected. This is perhaps not a matter for very great concern

in the case of individual laboratories, where the animals in different experiments concerned with the ætiology of cancer have been treated with the same tar and the results obtained are therefore directly comparable. It is, however, a matter of some difficulty to correlate the results of one laboratory with those of another where a tar of different carcinogenicity has been employed. Further, while it is a comparatively simple matter to induce skin tumours in the mouse, other animals are highly resistant to the action of coal tar. It is possible that treatment with a tar or other carcinogenic agent, many times as potent as ordinary coal tar, would induce such tumours in these animals. If skin tumours could be readily produced, for instance, in the rat or guinea-pig, the field of investigation into the ætiology of cancer would be appreciably enlarged. A simple method which would enable supplies of a standard type of tar of uniformly high carcinogenicity but low toxicity to be readily produced would be of value. In searching for such a method, that originally used by Kennaway (1924), who heated isoprene to 820° C., and later used by Twort and Ing (1928), who heated pinene in a special type of electric furnace, seemed the most promising. Through the courtesy of the staff of the Fuel Technology Department of this University, it has been possible to adapt a simple type of apparatus for the preparation of one of these high-temperature tars from purified turpentine. This apparatus has been in use in these laboratories for over a year, and has given good yields of a tar of low toxicity but high carcinogenicity.

DESCRIPTION OF APPARATUS.*

The apparatus is similar to that employed by Hague and Wheeler (1929) in their work on the pyrolysis of the paraffins, and the following description is taken in part from the original paper. The apparatus consists of a graduated glass burette B, a heating tube T and three condensers, C_1, C_2 and C_3, all in series. The graduated glass burette is fitted at the lower end with a tap T_1, and connected by a ground glass-silica joint to the rest of the apparatus. An inlet tube 1 allows a stream of nitrogen to be passed through the apparatus during the preparation of the tar. The tube T is of transparant silica, 70 cm. in length and 2·2 cm. internal diameter. Along its axis passes a narrow tube S, also of silica, whose closed end is situated just below the middle of the tube T. The tube S acts as a sheath for a thermocouple, by means of which the temperature at the middle of the tube S can be observed. A small silica flange L on the thermocouple sheath S ensures that the turpentine, if not vaporized previously, will do so on the flange. Thus the liquid from the burette passes through the tap T_1 down the silica tube and is projected on to the thermocouple tube S, down which it flows to vaporize on L. The silica tube is heated in an electric furnace F to a temperature of 850° C. The condensers are of glass, and to avoid their becoming blocked up, are so designed that the gas passes down the outside part and escapes up the middle tube of each condenser. Attached to each condenser by a ground-glass joint is a small receiver (R_1, R_2 and R_3). It is unnecessary to cool artificially the condensers and receivers,

* I am indebted to the Department of Fuel Technology of Sheffield University for the loan of the apparatus, and to Mr. R. A. Mott, M.Sc., F.I.C., and the Staff of the Department for much help and advice.

since the low boiling fractions of the tar formed by the decomposition of the turpentine are not required. Fig. 1 shows a diagram of the apparatus, which is adapted from that given in the original paper by Hague and Wheeler.

METHOD OF PREPARING THE TAR.

A stream of nitrogen is passed through the apparatus by means of the inlet tube 1, and the reaction tube T is heated to a temperature of 850° C. in the electric furnace. The burette B is filled with the purified turpentine, and the tap T_1 is so adjusted that the flow of liquid into the tube T is approximately ½ c.c. per minute. The rate of nitrogen flow is adjusted so that approximately 10 litres per hour pass through the apparatus. The products of decomposition are swept through the tube by the current of nitrogen, the liquid products are collected in the receivers R_1, R_2 and R_3, and the residual combustible gases are burnt at the outlet tube O. The tap T_1 requires a certain amount of readjustment from time to time as the head of liquid in the burette changes. Apart from this the apparatus is practically self-operating until the tube and condensers become choked up with carbon, etc. With the particular tube used in the present work, and allowing for a flow of turpentine of ½ c.c. per minute, the apparatus works for about four to five hours before becoming blocked up. During this time the yield of tarry products is about 7·5 c.c. per hour from every 30 c.c. of turpentine admitted into the apparatus. When the tube becomes blocked, the flow of nitrogen and turpentine is stopped, the furnace allowed to cool and the condensers and receivers disconnected. Gross particles of carbon are removed from the sides of the tube by means of a wire brush, and the tube is then reheated in the furnace, air being drawn through at the lower end until the tube is completely free from adhering carbon. If necessary, the carbon, which forms a ring at the lower end of the tube, can be removed by heating the tube in a blow-pipe flame. The tarry products in the receivers are drained into a flask and any adhering tar removed from the receivers by means of chloroform. The apparatus can now be connected up again, the nitrogen turned on and fresh supplies of purified turpentine admitted into the heated tube. After the apparatus has been working for several days and 100–200 c.c. of the mixture are available, removal of certain inactive constituents is carried out as follows:

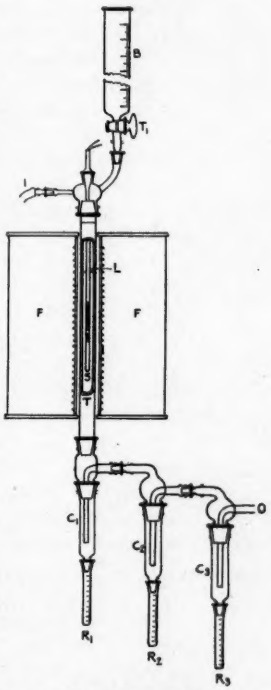

FIG. 1.—The Hague-Wheeler apparatus adapted for the preparation of potent carcinogenic tars.

Treatment of tar-chloroform mixture.—The mixture of tar and chloroform is allowed to settle for some days and is then filtered free from particles of carbon, and the filtrate is subjected to steam distillation. The distillate consists of

chloroform and low boiling fractions from the tar, but the chief product appears to be naphthalene. As soon as all but traces of this latter substance have come over, the distillation is stopped. In practice it is a matter of some difficulty to remove the naphthalene completely, and in order to obtain as standardized a material as possible under easily reproducible conditions, the alternative method of discontinuing the steam distillation as soon as the first traces of naphthalene appear may prove the more valuable one. The presence of the naphthalene probably has no appreciable effect on the carcinogenic activity of the tar, and by limiting the steam distillation in this way a further advantage is gained, in so much as the tar is less viscous and therefore more satisfactory for the skin treatment of the test mice. Finally the mixture of steam-distilled tar and water is poured into a separating funnel and left overnight, after which the tar is carefully freed from water.

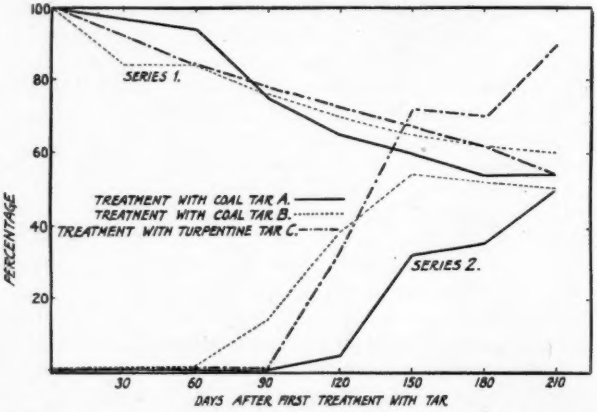

FIG. 2.—Percentage of survival rates of animals (Series 1). Percentage of animals with skin tumours (Series 2).

THE REACTION OF MICE TO THE TAR PREPARED BY THE PYROLYSIS OF PURIFIED TURPENTINE AT 850° C. IN THE HAGUE-WHEELER APPARATUS.

The relatively high potency of the tar prepared by the pyrolysis of purified turpentine at 850° C. in the Hague-Wheeler apparatus is illustrated in the following experiment :

Three groups each of 37 male mice were treated twice weekly in the interscapular region with (1) a coal tar A from a local gas-works (D 60 A), (2) a coal-tar B from a second gas works (D. 60 B), and (3) the tar prepared in the Hague-Wheeler apparatus and steam distilled until all but traces of the naphthalene have disappeared, under the conditions given above (D. 60 C). Coal-tar A was a mixed product of vertical and inclined type of retorts and B was a horizontal retort tar. The animals were maintained on a diet of 4 parts bread, 1 part Sussex ground oats and 1 part cabbage, made into a paste with

water, fed daily and supplemented once per week with cod-liver oil and marmite. The treatment with each of the tars was continued for three months, and the animals then kept under observation for a further period of five months. The relevant experimental data were collected and dealt with as described in a previous paper (Watson and Mellanby, 1930).

Fig. 2 shows the relative incidence of skin tumours and the survival rates of the animals in each of the three tar-treated groups.

Although the survival rate curves are very similar in type, the toxicity of the turpentine tar was least, and that of tar A greatest, when considered from the point of view of the animals surviving during the period following the end of the tar treatment. Throughout the experiment the superior condition of the mice treated with the turpentine tar was also shown by their improved appearance and relatively greater weight increases.

There was a striking difference between the incidence of skin tumours in the three groups. No tumours appeared until the 90th day after the first treatment with the turpentine tar (Group C), but within 60 days after this 72 per cent. of the surviving animals developed skin tumours which, with one or two exceptions, rapidly developed malignant characteristics. By the 210th day, 90 per cent. of the surviving animals had developed skin tumours. Tumours appeared slightly earlier in the animals treated with coal tar B, but a smaller percentage as a whole developed tumours. Thus, by the 150th day, 54 per cent. of the surviving animals had developed tumours, compared with 72 per cent. of the animals in the turpentine tar-treated group. Further, the early and almost universal development of malignant characteristics which was a feature of the tumours of the turpentine tar-treated group was not found in Group B. The appearance of skin tumours in the group treated with coal tar A was somewhat delayed, and by the 150th day only 32 per cent. of the surviving animals had developed tumours. It will be seen from Fig. 2 that the periods of time taken for 50 per cent. of the surviving animals in the three groups, A, B and C, to develop skin tumours were 210, 142 and 133 days respectively.

The difference in the potencies of the three tars is shown still more strikingly when we compare the rates of growth of the corresponding skin tumours over a period of some months (Fig. 3). The tumours of the turpentine tar-treated group (D. 60 C) grew more quickly and reached a greater size than those of either the other two groups. The pyrolysis of purified turpentine at 850° C. in the Hague-Wheeler apparatus results, therefore, in the formation of a tar of higher potency than is usually found in gas works coal-tars. This observation confirms the experiments of Twort and Ing (1928), who prepared a similarly potent agent from pinene. It is probable that these tars are among the most highly carcinogenic substances produced up to the present.

SUMMARY.

1. An apparatus originally described by Hague and Wheeler (1929) for their work on the pyrolysis of the paraffins has been adapted for the preparation from purified turpentine of a carcinogenic agent under standard conditions of temperature, etc.

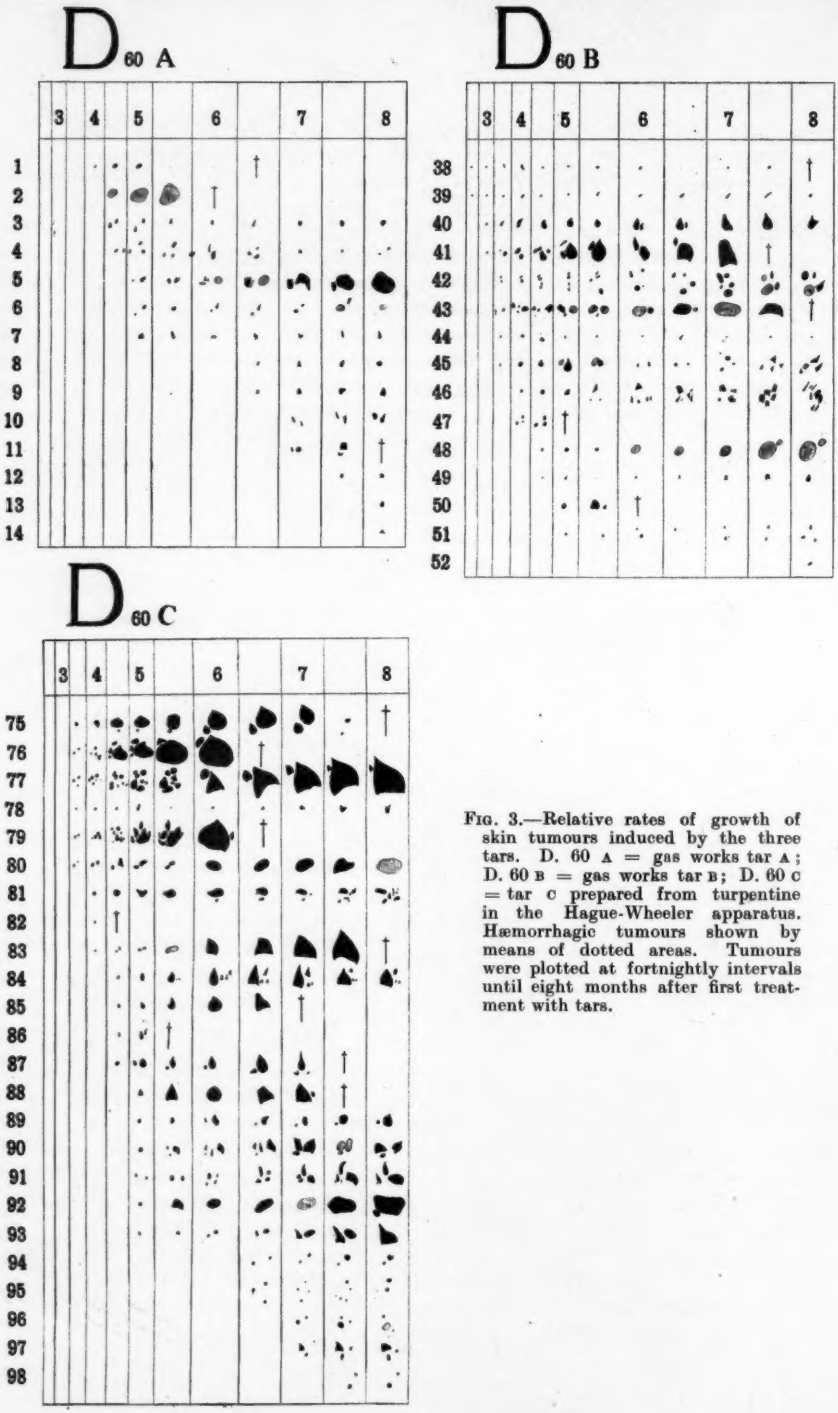

FIG. 3.—Relative rates of growth of skin tumours induced by the three tars. D. 60 A = gas works tar A; D. 60 B = gas works tar B; D. 60 C = tar C prepared from turpentine in the Hague-Wheeler apparatus. Hæmorrhagic tumours shown by means of dotted areas. Tumours were plotted at fortnightly intervals until eight months after first treatment with tars.

2. The pyrolysis of purified turpentine in this apparatus at 850° C. yields a tar which, after steam distillation, exhibits a high carcinogenicity combined with a low toxicity when tested on mice.

3. Charts showing the relative effectiveness of the tar prepared in this apparatus, which is simple in type and control, compared with two coal-gas tars, are included.

I am indebted to Prof. E. Mellanby, F.R.S., for his interest in the work. The expenses of the investigation were defrayed by the Yorkshire Cancer Research Council. Acknowledgments are also made to the University of London for the Laura de Saliceto Research Studentship, which was held during the course of the work.

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A QUANTITATIVE ANALYSIS OF THE LETHAL POWER OF A STRAIN OF TYPE I PNEUMOCOCCUS.

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THE data presented here have been abstracted from records of experiments we have been carrying out during the past two years on the titration of anti-pneumococcus serum (Type I). Although the provision of a standard serum will greatly facilitate the titration of antipneumococcus sera, the accuracy of the test is likely to be increased and the methods of titration simplified if the lethal power of the test culture can be maintained at a reasonably constant level, and if strains used by different workers as test cultures can be compared in this respect. There are no generally accepted criteria of lethal action available at the present time, so far as we are aware ; the analysis which follows is put forward as a contribution to the subject.

Lockhart (1926), Topley (1927) and Trevan (1927) have stressed the importance of studying problems of this kind by carrying out experiments on a scale which is adequate to eliminate as far as possible sampling errors that are inherent in small groups of observations. We have constantly tried to guard against this source of error, and are convinced of the necessity for doing so.

The estimation of the lethal power of a virulent bacterial culture which kills by multiplication in the tissues of the host is clearly not so simple a matter as the determination of the lethal dose of a bacterial toxin. Topley believes that the estimation of the average lethal dose of two bacterial strains requires several hundred animals for each comparative test, and Trevan (1930) agrees that the determination of a lethal dose for any given culture of pneumococcus is subject to an error of astronomical dimensions.

Felton (1930) and other workers have discussed the variable factors which tend to complicate the titration of a culture and of the corresponding specific serum, and among these factors variations in susceptibility of the mice have engaged their attention.

TECHNIQUE OF THE EXPERIMENTS.

The same strain of pneumococcus was used throughout the experiments, and the virulence was maintained by means of a weekly passage, 114 in all, through mice. For convenience of description this strain is referred to in the text and the tables as the " standard " culture.

Most of the mice used in our experiments were obtained from outside breeders. In the early stages of the work inbred mice from separate sources were used in individual experiments, but later, when results were obtained which indicated unequal resistance of different stocks, only mixed batches of mice were employed. The method of ensuring random selection of the mice was to place approximately equal numbers from different sources in a large cage and allow them to mix naturally for 10 or 15 minutes, when they were picked out, weighed, and put into the separate compartments of the experimental boxes. This procedure was generally carried out 24 hours before the experiment was begun, in order to observe the health of each mouse before it received the test-dose of culture. During the period of observation the mice received an ample and measured ration of food, which consisted of mixed grain and a small piece of fresh green cabbage. The mice received no food on the morning of the experiment. Mice between 6 and 8 weeks old were chosen; they weighed 15–20 gm. The mouse-boxes used during the tests were made of wood and measured approximately 4 in. × 4 in. × 40 in., each box being subdivided into ten compartments measuring about 4 in. × 4 in. × 4 in.—a convenient arrangement, because we worked with groups of 10 or multiples of 10 mice. Each compartment had a separate lid which could be readily removed for the purpose of observation. The strip of wood constituting the bottom of the whole box was easily detachable, and thus the cleaning of the boxes at the end of the experiment was readily accomplished. The segregation of the mice in separate compartments has the advantage that the animals, when seriously ill, are not disturbed in any way—thus rendering possible the observation of an accurate death-time. Cannibalism has frequently been noted in mice when

kept in groups, and there is consequently a risk of uninfected mice acquiring infection in this way.

All injections were made into the peritoneal cavity with a short guarded needle, so as to minimize the risk of injury to the viscera and at the same time to ensure a peritoneal inoculation; for mice are much less susceptible to a virulent pneumococcus culture when it is injected subcutaneously. All syringes and apparatus used for making the culture dilutions were standardized and known to be accurate to $\pm$ 2 per cent. After receiving the injection each mouse was returned to its compartment and was observed every four hours for a period of 100 hours. On rare occasions a mouse dies within the first four hours; such deaths are due to internal hæmorrhage, and are not included in the results. After a little practice the early signs of illness are recognizable without much difficulty, and at this stage the mouse is usually entered in the records as "ill"; if the course of the infection proves normal, several periods of "ill" and "very ill" follow, and finally death is recorded with a maximum error of four hours. An early symptom is the "roughening" of the normally sleek coat. As the infection develops the breathing becomes laboured, a mucous discharge is passed from the anus and frequently there is a purulent conjunctivitis. The animal remains in one position without movement and gradually passes into a state of coma, which terminates, in the course of a few hours, in death. Dyspnœa is undoubtedly the most characteristic symptom. It is essential that each mouse, while being observed, should be well illuminated, because otherwise the early symptoms of illness are likely to be missed.

CONTROLLABLE FACTORS WHICH MAY INFLUENCE THE COURSE OF THE INFECTION.

These may be considered as influencing (1) the infective agent and (2) the receptive test animal.

Factors which Influence the Lethal Power of the Test Culture.

An eighteen-hour broth culture of the standard strain is made by means of a heavy inoculation of material from the primary mouse-passage culture. Sterile broth, previously warmed in the incubator, is used as the diluting fluid, and the volumes of primary culture and of its dilutions are transferred by means of sterile 1 c.c. Ostwald pipettes, which, after emptying, are washed out twice in the dilution fluid. The dose is 1 c.c. of the appropriate dilution measured in the syringe. Table I illustrates the powerfully lethal effect of saline when used as a diluting fluid, even after 5 minutes' exposure of the cocci to its action; tyrode solution has a less harmful action than saline, and undiluted broth is evidently as satisfactory as any of the solutions we tested. Table II shows that the volume of the dose, whether 1 c.c. or 0·25 c.c. and the concentration of the broth, whether undiluted or 1 per cent. in saline, does not materially influence the resulting mortality in the mice.

TABLE I.—*Showing the Influence of Various Dilution Fluids—Saline, Tyrode Solution and Broth—upon the Lethal Power of a Virulent Strain of Type I Pneumococcus.*

Treatment of test culture.		Number of deaths within 100 hours	Percentage of deaths within 100 hours.	Mean death-time (hours).
Dilution fluid.	Time of exposure at room temperature before inoculation.	Number inoculated with $1/(100 \times 10^6)$ c.c.		
Saline	1 hour	1/30	3·3	62
,,	5 min.	5/20	25	48
Tyrode solution	1 hour	2/10	20	40
1% broth 10% broth	1 ,,	9/20	45	49
Undiluted broth	1 ,,	21/30	70	57

TABLE II.—*Indicating that in Virulence Tests of the Pneumococcus (Type I) the Volume of the Inoculum and the Concentration of Broth used as the Diluting Fluid do not Materially Influence the Mortality.*

Dilution of culture.	Diluting fluid.	Volume of dilution inoculated (c.c.).	Number of deaths within 100 hours Number inoculated.	Mean death-time (hours).
$1/(10 \times 10^6)$	Whole broth	1·0	10/10	52·2
,,	,, ,,	0·25	10/10	50·4
,,	1% broth in saline	1·0	10/10	40·2
,,	,, ,, ,,	0·25	9/10	38·5
$1(100 \times 10^6)$	Whole broth	1·0	10/10	47·8
,,	,, ,,	0·25	10/10	57·8
,,	1% broth in saline	1·0	8/10	46·5
,,	,, ,, ,,	0·25	9/10	47·9

The medium is an important factor in determining the lethal effect of the culture upon mice. Details of the media used in this work and the resulting mortality-rates will be found in Table III. Difco proteose peptone was invariably used, and indeed the medium was in every case similar to that used in the production of diphtheria toxin. The best medium (No. 8 in Table III) consisted of a horse-meat infusion with 2 per cent. proteose peptone, which was sterilized by Berkefeld filtration and subsequent steaming for one hour. A dose of $1/(100 \times 10^6)$ c.c. of the standard culture, when grown in this medium, killed 91 per cent. of 129 mice, with a mean death-time of 49 hours.

The lethal power of a virulent pneumococcus culture is compounded of two factors: (1) The density of the culture, that is, the number of virulent diplococci contained in 1 c.c. and (2) the virulence of the strain. It might be expected that with proper precautions for maintaining the virulence at a maximum the density factor is the more important one. A few observations on the density of virulent pneumococcus cultures are on record. Thus, Avery, Chickering, Cole and Dochez (1917) and, independently, Odell (1930) give an estimate of 100–300 million per c.c.; Gaskell (1927), 200–500 million per c.c.;

TABLE III.—*Comparing the Lethal Effect of the Standard Culture of Type I Pneumococcus when Grown in Different Media.*

Batch No. of medium used for standard culture	Date of preparation.	Composition of medium.	Mortality in mice inoculated with 1(100×10^6) c.c. of the standard culture.		Mean death-time (hours).
			Numbers.	Percentage.	
1	4.iii.30	2% Difco proteose broth used for production of diphtheria toxin. Autoclaved	25/29	86	45
2	9.v.30	Ditto, + 5% horse-serum	58/90	64	46
3	19.vi.30	As in batch No. 1	46/55	84	47
4	16.vii.30	Ditto	39/67	58	45
5	29.x.30	,,	157/203	77	54
6	11.ii.31	,,	26/55	47	54
7	3.iii.31	Horse-meat infusion + 2% Difco proteose. Seitz filtration; heated 58°, 1 hour, twice	81/104	78	56
8	27.v.31	Horse-meat infusion + 2% Difco proteose Berkefeld filtration; steamed 1 hour	117/129	91	49

and Felton (1930), 1000–2000 million per c.c. We are indebted to Dr. K. Iwaszkiewicz and Dr. J. Neyman, who happened to be engaged upon a similar problem, for examining the mortality data we obtained in groups of mice which had been inoculated with graduated doses of one of our test cultures; this was given in a beef-infusion broth. They (1931) estimated the density of this culture by means of statistical methods, and found it to be equivalent to 297·3 million infective units per c.c.; they concluded that the infective unit was one diplococcus. By means of colony counts in agar plates we have obtained the following figures as multiples of one million per c.c.: 695 (with a standard deviation of $\pm$ 49); 970 ± 81; these figures represent the mean from 20 plates; 745 ± 90; 820 ± 90; 330 ± 25; and 265 ± 36. Each of the last four figures represents the mean of the counts from 10 plates. The highest counts were given by horse-meat infusion media, and the lowest counts by a beef-infusion medium. An approximate average of our figures may be taken to be 500×10^6 diplococci per c.c.

The Question whether Apparent Irregularities in Tests for Lethal Power are due to Variations in Susceptibility of the Mice.

The Methods of Analysis of the Results of Infection in the Mice.

As we have already explained, 4-hourly observations were kept upon the mice during the period of 100 hours after the inoculation of the dose of culture.

The death-time was judged to be the mid-point of the time-interval between the hour when the mouse was last observed to be alive and the hour when it was noted to be dead ; the mean death-time of a group of mice may thus be assumed to be correct to within two hours ; groups of at least 10 mice were used for each dose of culture. The incubation period and the period of illness were similarly calculated.

The Source of the Mice.

Table IV gives the results obtained with two inbred races from different sources. Of 100 mice from Source A the mortality was 79 per cent. and the mean death-time 46 hours, when given a dose of $1/(100 \times 10^6)$ c.c. of the standard culture. The corresponding figures for 149 mice from Source B

TABLE IV.—*Indicating Differences in Susceptibility to a Virulent Type I Pneumococcus of Two Inbred Races of Mice from Independent Sources ; the Corresponding Figures for a Group of Mice from Mixed Sources are given for Comparison.*

Source of mice.	Number inoculated with $1/(100 \times 10^6)$ c.c. of standard culture.	Number of deaths within 100 hours.	Percentage mortality.	Mean death-time (hours).
A	100	79	79	46
Mixed sources	769	578	75	51
B	149	101	68	56

were 68 per cent. and 56 hours. We find the difference in the mortality to be possibly just significant ($\frac{d}{\sigma d} = 2{\cdot}0$). The results of a large group of mice from mixed sources are also given in Table IV ; they occupy an intermediate position between the other groups.

The Nutrition of the Mice Immediately Prior to the Injection.

Of 79 mice which were kept amply supplied with food, 53 died (67·1 per cent.) when given $1/(100 \times 10^6)$ c.c. of the standard culture, whereas of 80 mice which had fasted for 24 hours, but had otherwise been treated similarly, 59 died (73·7 per cent.)—a difference which is statistically insignificant. This result agrees with recent work by Neufeld and Etinger-Tulczynska (1931) on the influence of starvation upon intra-nasal infection with virulent pneumococcus cultures.

The Sex of the Mice.

Table V contains an analysis of data bearing on this point, and shows that the mean time of survival of male and female mice, when given equal doses of the virulent culture, is almost identical—a result which is consistent with the belief that there is no sex-differentiation in respect of susceptibility.

TABLE V.—*Indicating that Male and Female Mice do not Differ Significantly in Their Susceptibility to a Virulent Type I Strain of Pneumococcus.*

Dose (c.c.)	Males.		Females.	
	Number dying within 100 hours.	Hours' survival.	Number dying within 100 hours.	Hours' survival.
$1/(8\times10^6)$	10	506	4	176
$1/(16\times10^6)$	17	733	23	1179
$1/(32\times10^6)$	19	855	28	1548
$1/(100\times10^6)$ to $1/(3200\times10^6)$	36	2184	13	689
Totals	82	4278	68	3592
Mean survival-time in hours	52		53	

The Influence of Weight of the Mice upon the Infection.

The records show that of 467 mice, each weighing 15–19 gm. inclusive, 57·2 per cent. died ; and of 147 mice in the weight-group 20–24 gm. inclusive, 58·6 per cent. died. The mean survival-time of 272 mice weighing 15–19 gm. was 51·0 hours, and that of 85 mice weighing 20–24 gm. was 58·3 hours—a difference which we find to be significant. The mice in these groups received a dose of the standard culture varying from $1/(100\times10^6)$ to $1/(3200\times10^6)$ c.c. The results indicate that the weight factor does not influence the mortality-rate, but that the heavier mice survive longer. It seems, then, that for the purpose of testing the lethal power of cultures with doses within the above-mentioned range, mice between the weights 15–24 gm. may be accepted.

THE PROBLEM OF THE EXISTENCE OF AN INNATE IMMUNITY TO PNEUMOCOCCAL INFECTION (TYPE I) IN MICE.

Evidence of Resistance Derived from an Analysis of the Type of the Infection.

In Table VI there will be found a classification of the types of infection in groups of mice which received from $1/(100\times10^6)$ c.c. to $1/(3200\times10^6)$ c.c. of the standard culture (that is, from 1 to about 5 diplococci). Among a total of 1052 mice which were inoculated 52·4 per cent. died within the time-limit of 100 hours ; in about 2·5 per cent. the illness began late—after 50 hours—and in 2 per cent. the illness was a prolonged one, with survival beyond the time-limit. Again, in a group of 540 mice which were inoculated, 4·5 per cent. survived the test in an apparently healthy condition, and yet, when killed and examined, yielded a culture of pneumococcus from the heart-blood. It should be stated that, in order to simplify the technique for this purpose, the heart was punctured through the chest-wall, and thus the heart-blood may have been " contaminated " with lung-juice—a circumstance that does not modify the significance of the cultural findings.

TABLE VI.—*Indicating Variations in Susceptibility of Mice to a Virulent Type I Strain of Pneumococcus, as shown by the Numbers of Infected Animals Dying at an Early and Late Stage of the Period of Observation, and by the Number Yielding a Culture of Pneumococcus after the Time-limit of the Test, although in a Healthy Condition.*

Dose (c.c.).	Number of mice inoculated.	Number that died within 100 hours.	Number with short late illness.		Number with prolonged illness surviving beyond 100 hours.	Number of healthy survivors yielding positive heart-blood culture expressed as a ratio of the total mice inoculated.
			Dying within 100 hours.	Surviving beyond 100 hours.		
$1/(100\times10^6)$	423	326	6/423	5/423	14/423	5/80
$1/(200\times10^6)$	80	52	2/80	0/80	1/80	2/40
$1/(400\times10^6)$	80	43	2/80	0/80	3/80	3/40
$1/(800\times10^6)$	110	49	0/110	2/110	2/110	3/70
$1/(1600\times10^6)$	159	46	1/159	5/159	1/159	5/120
$1/(3200\times10^6)$	200	35	0/200	4/200	1/200	6/190
Totals	1052	551	11/1052	16/1052	22/1052	24/540
		52·4%	1·0%	1·5%	2·1%	4·5%

These data afford some evidence that a proportion of the mice possessed an unusual degree of resistance to the infection. It might be argued that some of the mice in this category received a dose of cocci which happened to be of

TABLE VII.—*Showing that Strains Isolated from Mice Surviving beyond the Time-limit of an Experiment in which only a Small Proportion Died within the Time-limit, nevertheless gave Evidence of High Virulence on Re-inoculation.*

No. of experiment.	Culture used in test. (The test-dose in each case = $1/(100 \times 10^6)$ c.c.)	Mortality-rate in mice.	Incubation period (hours).	Death-time (hours).
47	Standard culture	3/10	Mouse A : 70	84
			,, B : 46	60
			,, C : 22	44
			,, D : 82	106
			,, E : 78	120 (killed)
50	Heart-blood culture from Mouse D	10/10	20	46
50	Heart-blood culture from Mouse E	8/10	20	53

low virulence and possibly of the rough avirulent type. Table VII condenses some observations which were made in order to throw light upon this point. Briefly stated, on one occasion the test for virulence of the standard culture proved to be exceptionally poor, with delayed illness in the few mice which became infected, and an explanation was sought by re-testing the cultures obtained from the heart-blood of the mice. The results gave no support to the idea that the standard culture had undergone a change to the rough type, since the derived cultures were fully virulent; it may be supposed that the original group of mice were unusually resistant.

When groups of mice are given an equal dose of high dilutions of a virulent culture which on the average is likely to contain one diplococcus only, the proportion of mice which actually receive a diplococcus progressively diminishes, in accordance with a well-known statistical law, as the dilution increases. This explains why some of the mice escape infection, but we have wondered whether the test culture may have contained a small proportion of rough variants, and whether some of the surviving mice may have received an avirulent diplococcus. In order to test this point we estimated the virulence of a number of cultures which were derived from the standard culture by inoculating a series of serum-broth tubes with 1 c.c. of a high dilution of the culture [$1/(800 \times 10^6)$, $1/(1600 \times 10^6)$ and $1/(3200 \times 10^6)$.] The tubes which yielded growth were presumably derived from a single diplococcus. We tested seven such cultures and found all of them to be virulent (Table VIII). This procedure is a laborious one, and we hope to investigate by simpler methods the question of the possible occurrence of rough variants in a young virulent test culture.

TABLE VIII.—*Showing the Results of Testing the Virulence of Cultures Derived from the Standard Virulent Test Culture and Presumably Originating from a Single Diplococcus, since they were Obtained from Broth Tubes Inoculated with* $1/(800\times10^6)$ *c.c. or less of the Standard Culture.*

Source of culture as indicated by the amount of standard culture sown into broth-tubes (c.c.).	Lethal power of $1/(100\times10^6)$ c.c. of standard test culture.		Lethal power of $1/(100\times10^6)$ c.c. of culture derived from standard culture.	
	Deaths within 100 hours / Number of mice inoculated.	Mean death-time (hours).	Deaths within 100 hours / Number of mice inoculated.	Mean death-time (hours).
$1/(1600\times10^6)$	14/20	61	2/10	64
$1/(800\times10^6)$			5/10	64
$1/(800\times10^6)$	2/10	69	3/10	64
$1/(1600\times10^6)$			5/10	60
$1/(3200\times10^6)$			10/10	53
$1/(3200\times10^6)$	15/19	68	8/10	43
$1/(3200\times10^6)$			6/10	77

The Measurement of the Degree of Resistance in Mice to a Virulent Strain of Pneumococcus.

The method we have used is to compare the proportion of tubes of serum-broth yielding growth and the proportion of mice dying with an acute pneumococcal infection within 100 hours, when both the culture-tubes and the mice are inoculated at the same time with precisely the same volume (1 c.c.) of the identical dilution of the standard culture. The serum-broth tubes represent

TABLE IX.—*Giving Data which Afford a Measure of the Resistance of Mice to Varying Doses of a Virulent Type I Strain of Pneumococcus by Comparison with the Growth in a Series of Culture Tubes Sown with the Same Amounts of the Same Dilution of the Culture.*

Number of experiments in group.	Volume of standard culture inoculated (c c.).	Serum-broth tubes.			Mice.		
		Number inoculated.	Number yielding growth.	Percentage positive.	Number inoculated.	Number of deaths within 100 hours.	Percentage of deaths within 100 hours.
6	$1/(100\times10^6)$	70	70	100	90	74	82
6	$1/(200\times10^6)$	60	53	88	69	53	77
6	$1/(400\times10^6)$	60	46	77	70	42	60
8	$1/(800\times10^6)$	90	51	57	99	48	48
8	$1/(1600\times10^6)$	170	66	39	149	45	30
6	$1/(3200\times10^6)$	220	43	19·5	200	35	17·5
Totals		670	329		677	297	
			49·1%			43·9%	

TABLE X.—*Giving Data Similar to those in Table IX, but Including Animals which gave Evidence of Resistance to the*

TABLE X.—*Giving Data Similar to those in Table IX, but Including Animals which gave Evidence of Resistance to the Infection by Surviving beyond the Time-limit of 100 Hours.*

Number of experiments in group.	Volume of standard culture inoculated.	Serum-broth tubes.			Mice.			Mice surviving beyond the time-limit (100 hours).			Mice.		
		Total inoculated.	Number yielding growth.	Percentage positive.	Total inoculated.	Number of deaths within 100 hours.	Percentage dying within 100 hours.	With typical illness; culture positive in some cases.	With late illness; culture positive.	Healthy survivors; culture positive.	Total inoculated.	Total infected.	Percentage infected.
2	$1/(100\times10^6)$	30	30	100	40	38	95	1	0	0	40	39	97
1	$1/(200\times10^6)$	10	10	100	15	13	87	1	0	1	15	15	100
2	$1/(400\times10^6)$	20	20	100	30	21	70	2	0	1	30	24	80
4	$1/(800\times10^6)$	50	30	60	60	34	57	2	0	3	60	39	65
4	$1/(1600\times10^6)$	130	56	43	110	32	29	2	4	4	110	42	38
4	$1/(3200\times10^6)$	200	40	20	180	32	17·8	2	3	4	180	41	22·8
Totals		440	186		435	170		10	7	13	435	200	
			42·2%			39·1%			30			45·9%	

TABLE XI.—*Showing the Relation between Dosage of the Standard Pneumococcus Culture and the Resulting Infection. The Relationship is Expressed in Terms of the Mortality-Rate, the Incubation Period, the Period of Illness and the Death-time.*

Volume of standard culture injected (c.c.).	Number of mice inoculated.	Percentage mortality.	Incubation period.			Period of illness.			Death time.		
			Group with greatest number.		Mean duration (hours).	Group with greatest number.		Mean duration (hours).	Group with greatest number.		Mean death-time (hours).
			Time-interval (hours).	Number of mice in group.		Time-interval (hours).	Number of mice in group.		Time-interval (hours).	Number of mice in group.	
1/(100)	10	100	7	10	7·0	14–18	8	16·4	21–25	8	24
1/(1000)	70	100	6–8	57	7·4	13–18	22	25·0	26–30	22	32
1/(10,000)	10	100	7	10	7·0	26	6	27·6	31–35	6	35
$1/(1\times10^6)$	10	100	15–19	9	17·0	18–22	6	20·8	36–40	5	38
$1/(10\times10^6)$	126	92·9	18–21	56	20·9	19–24	20	30·0	38–42	29	47
$1/(16\times10^6)$	40	100	13–16	28	16·3	25–30	12	31·4	41–45	13	48
$1/(32\times10^6)$	50	94·0	17–20	26	20·7	25–30	14	30·4	41–45	16	51
$1/(50\times10^6)$	20	85·0	21–24	12	20·3	25–30	6	28·1	41–45	6	48
$1/(100\times10^6)$	769	75·2	17–20	116*	24·1	25–30	73*	29·2	41–45	128	51
$1/(200\times10^6)$	113	67·3	17–20	23	24·1	25–30	10	34·8	41–45	15	57
$1/(400\times10^6)$	95	53·7	17–20	15	23·3	25–30	8	31·5	41–45	10	54
$1/(800\times10^6)$	126	43·6	17–20	15	25·6	†	12	38·2	41–45	8	58
$1/(1600\times10^6)$	183	27·3	17–20	17	26·0	25–30	11	29·4	41–45	17	55
$1/(3200\times10^6)$	200	17·5	17–20	15	25·1	25–30	11	30·9	46–50	11	56

* The total number of mice observed was 350.

† Doubtful owing to irregular distribution.

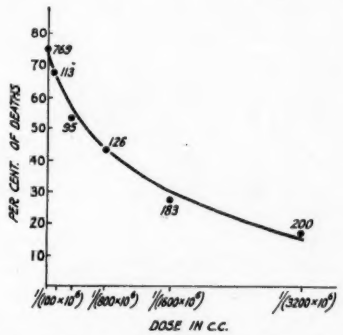
the most suitable medium we know for the growth of the pneumococcus, and it may be supposed that the individual tubes of the culture medium offer the same environment for the growth of the coccus. The results of comparative tests of this kind are assembled in Table IX, and it will be seen that 49 per cent. of the total serum-broth tubes yielded growth and that 44 per cent. of the total mice died within 100 hours. In spite of the numerous pipettings of sterile broth and of culture dilutions involved in these experiments, only 2·5 per cent. of the culture tubes showed contamination; these did not affect the accuracy of the observations. Table X contains similar data, but includes, in addition to the "acute" deaths among the inoculated mice, an analysis of those mice which survived beyond the time-limit of 100 hours with evidence of infection, thus making it possible to compare the proportion of culture tubes yielding growth with the total mice showing evidence of infection. It seems that about 7 per cent. of the mice which received an infecting dose possessed some resistance. The figures indicate that the proportion of positive tubes and mice, inclusive of all types of infection, is not very different; the slightly higher percentage in the group of total infected mice is apparently due to sampling errors. We may conclude that, under the conditions of this group of experiments, namely, the administration of a dose of from 1 to 5 virulent pneumococci, a small percentage of mice give evidence of an unusual degree of resistance. This conclusion is not necessarily applicable to a test-dose of the culture such as is used in the titration of sera, which may contain thousands of virulent diplococci.

THE RELATION BETWEEN DOSAGE OF A VIRULENT TYPE I PNEUMOCOCCUS AND THE RESULTING INFECTION.

The relevant data are collected in Table XI; this represents an analysis of 1822 mice, which were inoculated in groups at varying times with different doses of the standard culture. As has already been explained, the mice were drawn from mixed sources, and since we suppose that the other conditions of the experiment, for example, the lethal power of the culture, were not exceptional, the data probably represent a set of average results. We think that, viewed as a whole, they indicate a consistency which is all the more notable when the small size of some of the groups is considered. The close correspondence between the data which define the course of the infection in mice that received doses within the range of $1/(3200\times10^6)$ c.c.–$1/(16\times10^6)$ c.c. strongly suggests that the numbers (1–50) of diplococci in doses within this range possess the same lethal action. With large doses, however, namely, from $1/(10\times10^6)$ c.c. to 1/100th c.c., the course of the infection, as judged by the incubation period, the period of illness and the death-time, exhibits a progressive acceleration. It seems, therefore, that the dose of $1/(10\times10^6)$ c.c. represents a critical level of lethal intensity. The numerical estimates of the density of our cultures, already given, indicate an average density of approximately 500×10^6 diplococci per c.c. It follows that most of the infected mice inoculated with doses less than $1/(400\times10^6)$ c.c. received a single diplococcus; the mice which received $1/(100\times10^6)$ c.c. were probably given about

5 diplococci; those which received $1/(10\times10^3)$ c.c., about 50 diplococci; and so on until the 1/100th c.c. dose; this contained approximately 5 million diplococci.

There is a high probability that the infected mice which were given the three smallest doses—$1/(800\times10^6)$ c.c., $1/(1600\times10^6)$ c.c., $1/(3200\times10^6)$ c.c.—were infected by one diplococcus. Reference to Table IX shows that of 480 serum-broth tubes inoculated with these amounts, 160 (33·3 per cent.) yielded a growth of pneumococcus, and that of 448 mice inoculated with identical doses 128 (28·6 per cent.) died, a result which suggests that 90–95 per cent. of mice receiving one diplococcus succumbed to an acute infection. A smooth virulent test-culture is composed mostly of diplococci, but contains from 10 to 15 per cent. of elements consisting of short chains. On the supposition—which is incapable of proof—that the chains are not broken up in the process of dilution, it is clear that diplococci constitute at least 80 per cent. of the infective units.



The characteristic curve showing the relation between the dose of the standard Type I pneumococcus culture and the resulting mortality. The figures at the points of the curve represent the numbers of mice used.

The question has sometimes been raised whether it is possible to estimate an average lethal dose of a bacterial culture. Both Topley and Trevan have expressed their doubts, and yet our experience leads us to think that it should be possible to determine an average (50 per cent.) lethal dose for a virulent pneumococcus culture. At an early stage of our work we chose a dose of $1/(100\times10^6)$ c.c. as a control to the virulence of the culture used in serum titrations, because we soon ascertained that it killed with constancy between the 50 per cent. and 100 per cent. levels. Table XI shows that the mortality among 769 mice which were inoculated with this dose is 75 per cent. The fluctuations in samples of 10 mice did not exceed the limits of errors of sampling, so far as we could judge. For this group, which is the largest in the Table, the mean incubation period is 24 hours, the mean death-time is 51 hours, and the time-interval containing the largest number of deaths is 41–45 hours. We believe that data of this nature are capable of specifying the lethal power of a virulent culture, so that comparison may be made with strains from any other source.

The mortality results, which are given in Table XI, when plotted, give a characteristic curve, from which an average lethal dose, that is, one which gives a 50 per cent. mortality, can be calculated; for our culture this is approximately $1/(500\times10^6)$ c.c. When the logarithm of the doses from $1/(16\times10^6)$ c.c. to $1/(3200\times10^6)$ c.c. is plotted against the corresponding percentage mortality, the points of the curve fall upon a straight line.

Finally, it seems that the limited degree of resistance of mice to a minimal dose of pneumococci and the regularity of the lethal tests are correlated with the circumstance that, as Neufeld has recently pointed out, mice do not suffer from spontaneous pneumococcal infections, and thus do not possess an acquired immunity.

SUMMARY.

The factors which influence the lethal power for mice of a virulent Type I pneumococcus culture have been investigated. The density and the virulence of the culture determine its lethal power. The controllable factors which influence the test animal have likewise been examined. Stocks of mice from different sources appear to show some variation in susceptibility. There is no sex factor, nor does the weight factor influence the mortality, although it modifies the survival-time. A small proportion of mice—from 5 to 10 per cent. —possess an innate resistance to small doses of pneumococci. A characteristic curve relating dosage to percentage mortality among mice has been obtained, and has furnished evidence that an average lethal dose of a culture can be calculated by the use of reasonable numbers of mice; this dose contains one diplococcus. The percentage mortality, the mean death-time, and the distribution of the deaths in appropriate time-intervals in a group of mice which receive a definite dose of a test culture, preferably of the order of $1/(100\times10^6)$ c.c. to $1/(500\times10^6)$ c.c., are criteria by means of which the lethal power of any culture of Type I pneumococcus may be specified.

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INDEX TO AUTHORS.

ALSTON, J. A., and GIBSON, H. J., a note on the experimental transmission of "louping ill" to mice, 82

——— and STEWART, D., some observations on passive immunity in the normal subject produced by antipneumococcic sera, 49

ARMSTRONG, R. R., serum treatment of lobar pneumonia; a study in immunology, 279

ASCHNER, M., see *Kligler, I. J.*

ATKIN, E. E., the inhibitory action of sanocrysin on tuberculous lesions, 249

BARR, M., and GLENNY, A. T., further observations on qualitative differences in antitoxic fractions prepared from the same sample of plasma, 337

——— ——— and POPE, C. G., some observations on the distribution of antitoxins in serum, 217

BARRY, D. T., see *Chauchard, A.*

BEDSON, S. P., immunization with killed herpes virus, 254

BLAND, J. O. W., glandular fever, II. The protozoal nature of the experimental disease, 311

BROOM, W. A., a note on the toxicity of amyl-meta-cresol, 327

BUCKLEY, O. B., the effect of subcutaneous injections of trypsin on the blood-sugar and on insulin action, 13

BURNET, F. M., and MACNAMARA, J., immunological differences between strains of poliomyelitic virus, 57

CAMPBELL, J. A., and FILDES, P., tetanus. X: The effect of the oxygen tension of the tissue fluids in controlling infection by *B. tetani*, 77

CHAUCHARD, A., CHAUCHARD, B., and BARRY, D. T., effects of hæmorrhage on the vascular nervous mechanism, 190

CHAUCHARD, B., see *Chauchard, A.*

CLEARKIN, P. A., and SKAN, D. A., the effect of repeated calf passage on the immunizing power of vaccine lymph, 322

COOPER, K. E., see *Gordon, J.*

COULTHARD, C. E., the disinfectant and antiseptic properties of amyl-meta-cresol, 331

COX, G. A., see *Drennan, A. M.*

CRADDOCK, S., see *Parish, H. J.*

——— and PARISH, H. J., the serum treatment of anaërobic (*Vibrion septique*) infections in mice, 389

CRAIGIE, J., a method of drying complement from the frozen state, 75

CUMMINS, S. L., and WEATHERALL, C., the retardation of lytic processes by colloidal silica solution, 239; effects of colloidal silica upon the growth of tubercle bacilli in blood, 245

CZARKOWSKA-GLADNEY, J., and HURST, E. W., some data concerning the infectivity, survival and powers of diffusion of the virus of "louping-ill," 426

DAU, H., see *Pijper, A.*

DON, C. S. D., see *Jenkins, C. E.*

DOWNIE, A. W., STENT, L., and WHITE, S. M., the bile solubility of pneumococcus, with special reference to the chemical structure of various bile-salts, 1

DRENNAN, A. M., MALCOLM, J., and COX, G. A., studies on rat thyroids, 430

DUDLEY, S. F., human skin-tolerance to diphtheria toxin, and immunizability, 319

EAGLES, G. H., see *McClean, D.*, and *Green, A. B.*

——— and MCCLEAN, D., cultivation of vaccinia virus in a cell-free medium, 97

EAVES, E. C., some observations on calcium and phosphorus in the brain in different conditions, 113

ELFORD, W. J., see *Galloway, I. A.*

FAIRBROTHER, R. W., see *Hurst, E. W.*

FILDES, P., see *Campbell, J. A.*

FINDLAY, G. M., the fractionation of anti-vaccinia serum, 9

FLOREY, H., the mechanism of goblet-cell secretion in the mammal; the effects of cyanide, 301

——— and WEBB, R. A., mucus secretion in acute experimental inflammation of the colon and other mucous membranes of the cat; histological changes, 286

GALLOWAY, I. A., and ELFORD, W. J., filtration of the virus of foot-and-mouth disease through a new series of graded collodion membranes, 407

GIBSON, H. J., see *Alston, J. A.*

GILDING, H. P., the relation of the nervous system to a patchy ischæmia (Bier's spots) in animals, 66

GILMORE, E. STG., complement-fixation with vaccinia culture virus, 165

GLENNY, A. T., see *Barr, M.*

——— HAMP, A. G., and LLEWELLYN-JONES, M., the absorption of diphtheria antitoxin, 21

GORDON, J., and COOPER, K. E., a study of the phosphorus distribution in bacterial cultures; Part II, 234

GREEN, A. B., and EAGLES, G. H., the filterability of vaccinia virus, 202

GYE, W. E., a note on the propagation of Fujinami's fowl myxo-sarcoma in ducks, 93

HAMP, A. G., see *Glenny, A. T.*

HAPPOLD, F. C., and TAYLOR, A., the lipolytic activity of tubercular guinea-pigs' tissues. III: Variation of activity with the spread of the disease, 272

HARE, R., studies on immunity to hæmolytic streptococci. III: Observations on the variations in resistance to phagocytosis displayed by broth cultures of strains of high and low virulence, 261.

HOLMES, B. E., see *Pirie, A.*

HURST, E. W., see *Czarkowska-Gladney, J.*

——— and FAIRBROTHER, R. W., simultaneous infection of the monkey with the viruses of poliomyelitis and vaccinia, 17

JENKINS, C. E., and DON, C. S. D., comparative test of the estimation of hæmoglobin by the flicker hæmoglobinometer and the oxygen capacity method, 212

KINNEAR, J., an investigation into the streptococci found in certain diseases of the skin, 384

KLIGLER, I. J., recovery of fowl-pox virus from vaccines by cataphoresis, 42

——— and ASCHNER, M., the relation between fowl-pox (epithelioma contagiosum) and avian diphtheria, 35; studies on protein-free suspensions of viruses. II: Cataphoresis experiments with protein-free suspensions of a bacteriophage and fowl-pox virus, 178

——— and OLITZKI, L., cataphoresis experiments with typhus virus, 69; studies on protein-free suspensions of viruses. I: The adsorption and elution of bacteriophage and fowl-pox virus, 172; III: The resistance to heat and disinfectants of protein-free eluates of a bacteriophage and fowl-pox virus, 393

LEDINGHAM, J. C. G., MORGAN, W. T. J., and PETRIE, G. F., the potency and distribution in the serum fractions of antiviral body obtained by immunization of the horse with vaccinia virus, 357

LLEWELLYN-JONES, M., see *Glenny, A. T.*

LUDFORD, R. J., resistance to the growth of transplantable tumours. I: The influence of vital staining on induced resistance, 45; II: The influence of dyestuffs and of colloids on natural resistance, 108

MCCLEAN, D., see *Eagles, G. H.*

——— and EAGLES, G. H., the conservation of vaccinia virus grown *in vitro*, 103

MACNAMARA, J., see *Burnet, F. M.*

MALCOLM, J., see *Drennan, A. M.*

MARRACK, J., and SMITH, F. C., quantitative aspects of immunity reactions: the precipitin reaction, 30; the composition of the precipitate in precipitin reactions, 182

MORGAN, W. T. J., a specific precipitating polysaccharide from *B. dysenteriæ* (Shiga), 62; see *Ledingham, J. C. G.*, and *Petrie, G. F.*

MOTTRAM, J. C., experiments on the production of tumours on the somatic mutation hypothesis, 378

OKELL, C. C., and PARISH, H. J., the effect of sodium aurothiosulphate in the treatment of tuberculosis in guinea-pigs, 136

OLITZKI, L., see *Kligler, I. J.*

O'MEARA, R. A. Q., the mechanism of the Voges-Proskauer reaction and the diacetyl reaction for proteins, 346

PARISH, H. J., see *Craddock, S.*, and *Okell, C. C.*

——— and CRADDOCK, S., a ringworm epizootic in mice, 209

PATTERSON, J., the preparation and standardization of colloidal gold for the Lange test, 143

PETRIE, G. F., see *Ledingham, J. C. G.*

——— and MORGAN, W. T. J., a quantitative analysis of the lethal power of a strain of Type I pneumococcus, 447

PIJPER, A., and DAU, H., agglutinins of tick-bite fever and sporadic typhus in Southern Africa in man and in guinea-pigs, 123

PIRIE, A., adsorption experiments with the Rous sarcoma virus, 373

——— and HOLMES, B. E., the cause of the inactivation of the Rous sarcoma filtrate during incubation, 127

PLATT, R., the blood glutathione in disease, 139

POPE, C. G., see *Barr, M.*

PURCHASE, H. S., a study of cross-immunity with viruses of fowl-plague and observations on the duration of immunity, 199

ROSHER, A. B., the effect of the X and V growth factors on the pathogenicity of indole-producing strains of influenza bacilli, 133

RUDD, G. V., observations on the acidity and chloride content of the gastric juice, II, 226

SKAN, D. A., see *Clearkin, P. A.*

SMITH, F. C., see *Marrack, J.*

SOKOLOVITCH, M., the effect of X-ray therapy on the partition of phosphorus compounds in the blood in disease, 147; a contribution on the distribution of calcium content in blood of children, 150

SPURRELL, W. R., experimental observations with low dose radiation, 402

STENT, L., see *Downie, A. W.*

STEWART, D., see *Alston, J. M.*

TAYLOR, A., see *Happold, F. C.*

WATSON, A. F., tar cancer in mice. III: An apparatus for the preparation under standard conditions of a highly potent carcinogenic agent of low toxicity, 441

WEATHERALL, C., see *Cummins, S. L.*

WEBB, R. A., see *Florey, H.*

WHITE, S. M., see *Downie, A. W.*

WHITEHEAD, R., the significance of natural variations in the structure and cortical lipoid of the mouse suprarenal, 305

WILSON, G. S., the gaseous requirements of *Br. abortus* (bovine type), 88; the growth of *Br. abortus* (bovine type)in shake tubes, 152